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FACTORS INFLUENCING CHOLESTEROL OXIDATION IN FOOD FATS AND POULTRY PRODUCTS

BY

XUEQIN SUECHIN LI

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

FOODS and NUTRITION

DEPARTMENT OF AGRICULTURAL, FOOD AND NUTRITIONAL SCIENCE

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Dr. J. S. Sim, Supervisor

Dr. W. C. Sauer

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Dr. S. R. Jeffrey

Dated: Aug. 25 1995

DEDICATION

To my husband Houshi for all his love and dedication and his patience and support

To my daughter Mandi, and my son Adam, who have brought a new ray of sunshine into my life

To my father Daoyan, and my mother Zhenni for their love and kindness and support

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ABSTRACT

Cholesterol in food fats undergoes autoxidation and forms cholesterol oxides, which are regarded as strong atherogenic agents in humans. Cholesterol oxidation is a free radical-mediated process which occurs during food storage and cooking, and with exposure to light and oxygen. The objective of this study was to assess the effect of storage time and cooking temperature on cholesterol oxidation of food oils and poultry product lipids, which have different fatty acid profiles, respectively. Natural tocopherols were tested as effective antioxidants in preventing cholesterol oxidation.

The effect of storage time and cooking temperature on cholesterol oxidation in fish, flax, sunflower and palm oils, to which cholesterol was added, was examined. The contents of cholesterol oxides in all oils increased ($P<0.05$) with time of storage. Food oils heated for 22 hr at 110 C generated the greatest amounts of cholesterol oxides. Addition of antioxidants to these oils effectively prevented or reduced cholesterol oxidation. Total cholesterol oxides in fish oil were higher ($P<0.05$) than that in the vegetable oils. The degree of natural tocopherol contents in the oils was flax oil > sunflower oil > palm oil > fish oil. Palm oil was the most stable during storage and cooking conditions.

Effects of feeding fish, flax, sunflower and palm oils, with and without tocopherol supplementation, to laying hens on cholesterol stability of resultant eggs and chicken meats were investigated. Spray-dried egg yolk and freeze-dried chicken meat preparations were subjected to storage and cooking. Tocopherol contents were rapidly depleted during the 4 month storage period, with concomitant increases of cholesterol oxides. The greatest

cholesterol oxidation occurred in the fish oil diet and the least in the palm oil diet. Heating egg yolk powder significantly accelerated cholesterol oxidation. Tocopherol supplementation significantly reduced cholesterol oxide formation respective of the types of dietary oils. Results suggest that cholesterol oxidation either in food oils or poultry products, are naturally generated under storage and cooking conditions but can be effectively prevented by supplementing with tocopherols.

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Chapter 1. General Introduction

Cholesterol autoxidation has been recognized and studied for the past 90 years, but it wasn't until the 1960's that a systematic study of cholesterol autoxidation was undertaken (Smith, 1981). More recently interest in the oxidation of this sterol has intensified, spurred by reports on adverse effects of cholesterol oxidation products on human health (Addis et al., 1983; Peng and Morin, 1992). Most studies of the biological effects of the oxides were carried out on animals. Cholesterol oxides created concerns about their effects on humans. Several biological effects have led researchers to speculate that a link may exist between ingested cholesterol oxidation products and coronary heart disease (Taylor et al., 1979). This notion was supported by the finding (Imai et al., 1976) that cholesterol oxides from USP (United States Pharmacopoeia) grade cholesterol produced angiotoxicity and arteriosclerosis in rabbits, while purified cholesterol did not.

Autoxidation of cholesterol, similar to that of other lipids, is enhanced by contact with oxygen (air) at elevated temperature, free radical initiators (such as metals, haem compounds and salt) and light (Hubbard et al, 1989). Foods high in cholesterol when exposed to heat, light, and/or air during storage and processing may be important sources of dietary cholesterol oxidation products associated with atherosclerosis. Much research has focused on developing foods with reduced amounts of cholesterol. Since all animal cells require cholesterol for vital functions, it is difficult and perhaps impossible to remove it completely (Rankin and Pike, 1993). Addis (1990) suggested, since there is no

practical method for lowering cholesterol in certain foods (like muscle tissue), that research should be directed to protecting cholesterol from oxidation.

1. 1. Cholesterol

Cholesterol was first described by a French chemist de Fourcroy who in 1789 isolated a crystalline substance from the alcohol-soluble fraction of human gallstones (de Fourcroy, 1789). The structure of cholesterol (Fig 1-1) was established in 1932 by Windaus (1932) and Wieland and Dane (1932). Cholesterol is almost ubiquitously distributed in the cells of all animals. In mammals, the largest amounts of cholesterol are contained in muscle, brain and nervous system, and connective tissues including adipose (Sabine, 1977; Gibbons et al., 1982). Cholesterol is present predominantly in the free or esterified forms, with a small proportion being sulphated or conjugated (Sabine, 1977). In all cells, except adipose tissue, the bulk of cellular cholesterol is contained in or associated with cellular membranes (Davison, 1970).

Cholesterol is an important precursor to a variety of key biological substances. It is converted in the liver into bile acids which are indispensable for the digestion and absorption of dietary lipids (Stryer, 1981). Vitamin D_3, steroid hormones such as progesterone, estrogens, androgens, glucocorticoids, cortisone, hydrocortisone, and aldosterone are synthesized from cholesterol, and are essential for normal sexual function, anti-inflammation, and sodium-regulation (Sabine, 1977; Block, 1985). Some precursor molecules of cholesterol are also necessary for *de novo* synthesis of biologically important compounds such as ubiquinones and dolichol (Block, 1985; Health and Welfare Canada,

1990). An essential compound of biological membranes, cholesterol influences membrane fluidity. Consequently, membrane permeability to water and solutes, membrane fusion, and the activities of membrane-bound enzymes, hormone and other receptors are altered by the presence of cholesterol (Sabine, 1977; Block, 1985).

1. 2. What are Cholesterol Oxidation Products (COPs)

Cholesterol is sensitive to oxidation forming cholesterol oxidation products (COPs). The cholesterol oxidation products possess biological activities such as sterol biosynthesis modulation, angiotoxicity, cytotoxicity, mutagenicity and carcinogenicity (Taylor et al., 1979; Smith, 1987). Over 60 products resulting from autoxidation, photo-oxidation and enzymatic action have been described (Smith, 1981). The major COPs in foodstuffs include the following: 25-hydroxycholesterol (25-OH), cholestan-3β-5α-6β-triol (triol), 5,6α-epoxy-cholesterol (α-epoxide), 5,6β-epoxycholesterol (β-epoxide), 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH), and 7-ketocholesterol (7-keto) (Finocchiaro and Richardson, 1983; Smith, 1987). Of these, 25-OH and triol have been shown to be the most toxic in cultured cell and experimental animal studies (Kandutsch et al., 1978; Peng et al., 1979; Imai et al., 1980; Ansari et al., 1982).

1. 3. Formation of Cholesterol Oxidation Products

The chemical structure of cholesterol is shown in Figure 1-1. The common features of these molecules are a polycyclic nucleus with four fused rings, a branched aliphatic side chain attached to the D ring at C-17, a hydroxyl group that is attached to

C-3 of the A ring and is β in configuration, and a Δ^5 double bond in the B ring. Of relevance to the question of autoxidation are the B ring unsaturation and positions allylic to it, and the presence of two tertiary carbons in the side chain. Cholesterol oxidation is initiated by hydrogen abstraction, predominantly at C-7 (Maerker, 1987). The types of oxidation products seem to depend upon the physical state of cholesterol. In aqueous colloidal solutions, the 5, 6-double bond in the A and B rings seems to be the most reactive site, whereas in the crystalline state in the presence of air, the point of attachment of oxygen is towards the tertiary 20 and 25 carbon positions (Peng and Taylor, 1984). In both reactions, there is a formation of oxidation products, each reaction yielding different compounds (Peng and Taylor, 1984). The most common COPs are shown in Figure 1-2. Oxidation in the side chain first yields a variety of hydroperoxides, which are decomposed and produces 25-hydroxycholesterol (25-OH) and 20α-hydroxycholesterol (20α-OH)and others. The allylic C-7 radical first reacts with oxygen to give the 7-peroxyl radicals, which form the epimeric 7α- and 7β-hydroxycholesterol and 7-ketocholesterol, which goes to cholesta-3, 5-dien-7-one. The epoxidation of the cholesterol may occur by attack of 7-hydroperoxides already formed, yielding isomeric α- and β-epoxides, which gives cholestane-3β, 5α, 6β-triol (triol) by hydration (Smith, 1987; Hubbard et al., 1989). Many researchers noted that cholesterol oxidation ceased when 70-75% of the substrate had been consumed. This effect may be caused by changes in micellar structure due to the presence of the oxidation products (Maerker, 1987). The solubility of cholesterol in aqueous media also is enhanced by cholesterol oxidation products (Krut, 1982).

1. 4. Biological Effects of Cholesterol Oxidation products (COPs)

Many COPs compounds possess undesired biological effects, not observed with pure cholesterol, at the enzymic or cellular level when animals were given a concentrated dose of cholesterol oxides (Peng et al., 1978; Imai et al., 1980; Smith, 1987). Once ingested, Cholesterol oxides can be absorbed through the intestinal tract and are selectively transported by LDL (low density lipoproteins) and VLDL (very low density lipoproteins) in the blood stream, exposing arterial tissue to their effects (Peng et al., 1982; Peng et al., 1987; Emanuel et al., 1991). COPs are extremely angiotoxic and have been implicated in the aetiology of atherosclerosis (Erickson et al., 1977; Kandutsch et al., 1978; Imai et al., 1976, 1980; Taylor et al., 1979; Peng and Taylor, 1984; Parish et al., 1986). Membrane alteration caused by the incorporation of the cholesterol oxides (instead of cholesterol) into the cellular membrane could, by virtue of their analogous structure, affect the membrane's barrier properties and lead to cell death (Herian et al., 1985). The dead cells could in turn be the primary area for lipid infiltration, leading to atherosclerosis (Nourooz-Zadeh and Appelqvist, 1989). This has been demonstrated in that small amounts of oxidized cholesterol products, such as 25-hydroxycholesterol and cholestane-triol, have been shown to induce arterial injury within 24 hr of administration to rabbits (Imai et al., 1976, 1980; Taylor et al., 1979; Jacobson et al., 1985). Moreover, human subjects fed a meal of powdered eggs that were high in COPs have exhibited increased levels of cholesterol oxides in chylomicrons and in total plasma (Emanuel et al., 1991; Howard et al., 1991). Similar cytotoxic effects were observed when COPs were incubated with rabbit arterial smooth muscle cells and human endothelial cells in culture

(Cox et al., 1988). The most potent cytotoxicity was elicited by 25-OH followed by triol (Cox et al., 1988). 7α- and 7β-hydroxycholesterol, cholestan-3,5-dien-7-one, 7-ketocholesterol and 5,6-epoxycholesterol also exhibit significant cytotoxicity (Taylor et al., 1979; Cox et al., 1988; Pie and Seillan, 1992). Certain cholesterol oxides, for example cholesterol epoxides, are also regarded as potent mutagenic or carcinogenic agents (Ames, 1983). α-epoxide has been reported to induce tumour formation in rats and mice after subcutaneous administration by both oil and aqueous vehicles (Bischoff, 1969), this compound has also been found to be present in ultraviolet irradiated human skin (Black and Lo, 1971; Black and Chen, 1976), and in the skin of mice (Black and Douglas, 1972). However, no information is available concerning the carcinogenicity of the 5β-,6β-epoxycholestanol isomer.

Clearly, the ingestion of COPs may have implications for human health, particularly in the long term, and detrimental effects on the blood-vascular system. It is difficult to speculate on the significance of cholesterol oxide-induced cytotoxicity as it relates to atherogenesis in human. However, metabolism of lipoproteins containing COPs may be affected by the presence of lipid oxidation products in addition to COPs themselves. Thus, various lipid oxidation products may act independently or in concert with one another to influence metabolic events associated with atherogenesis (Emanuel et al., 1991).

1. 5. Relationship between Lipid Oxidation and Cholesterol Oxidation

Smith(1980) indicated that cholesterol autoxidation was a free radical mechanism, similar to autoxidation of unsaturated fatty acids (Nawar, 1985). Oxidation of unsaturated fatty acids proceeds through a free-radical chain mechanism involving initiation, propagation, and termination steps (Figure 1-3). Polyunsaturated fatty acids (PUFAs), especially 20:5n3 (EPA) and 22:6n3 (DHA) are markedly susceptible to peroxidation, even under mild ambient conditions, and they are easily incorporated into the accepted chain mechanism of lipid peroxidation to yield free radicals and peroxy radicals (Hsieh and Kinsella, 1986; Frankel, 1987). Therefore, oxidation of cholesterol might also be accelerated in the presence of peroxidized lipids (Ohshima et al., 1993; Li et al., 1994)

The beneficial effects of dietary n-3 PUFA include reducing plasma triglyceride (TG), total cholesterol (TC), and low density lipoprotein-cholesterol (LDL-C); increasing high density liporotein-cholesterol (HDL-C); decreasing platelet aggregation, blood pressure, blood viscosity and fibrinogen; replacing arachidonic acid (AA) in the cellular phospholipid pool, inhibiting the syntheses of eicosanoids, such as prostaglandin E_2 (PGE_2) and thromboxane (TXA_2) (Kinsella et al., 1990; Simopoulos, 1991). In addition, essentiality of dietary n-3 PUFA in the growth and development of animals has recently been established (Simopoulos, 1991). Dietary n-3 fatty acids, particularly eicosapentaenoic acid (EPA, 20:5n3) and decosahexaenoic (DHA, 22:6n3) modulate numerous physiological functions crucial to human health (Leaf and Weber, 1988). Singh and Chandra (1988) reviewed evidence of increasing requirements for n-3 fatty acids in low n-3 fatty acids diet for human. The high rate of diet-related cardiovascular deaths recorded

in the American population, when compared to Japanese and Eskimos populations may be due to differences in the ratio of n-6 to n-3 fatty acids in their diets (Yamori et al., 1985; Kinsella 1990). These findings stimulated research on how to ensure an adequate dietary supply of omega-3 fatty acids for humans. The enrichment of chicken egg (Sim, 1990) and chicken meat (Ajuyah et al., 1991a, 1991b) with omega-3 fatty acids by incorporation of flax and canola seeds (or their oils) in laying hen diets has been well documented. However, substantial amounts of omega-3 fatty acids in poultry products decrease saturation and could enhance lipid oxidation. Lipid oxidation in meat, egg and their products has been shown to be highly correlated to the degree of unsaturation or the number of double bonds in the fatty acids (Nawar and Hultin, 1988). Monounsaturated fatty acids are far less susceptible to lipid autoxidation than polyunsaturated fatty acids and relative rates for methyl oleate (C18:1n9), linoleate (C18:2n6) and linolenate (C18:3n3) at 20°C are 1:12:25, respectively. Moreover, Fritsche and Johnston (1988) identified the following as possible health risks associated with increased consumption of omega-3 fatty acids: 1) increased consumption of lipid oxidation products, 2) increased in vivo production of lipid oxidation products, and 3) depletion of tissue levels of vitamin E.

1. 6. COPs in Foods

In North America, animal fat accounts for approximately 57% of total fat available for consumption. In Canada, the average intake of cholesterol is about 440 mg per day per person (Health and Welfare Canada, 1990). Cholesterol is an unsaturated compound

and is readily autoxidized to form cholesterol oxidation products. These COPS are formed in cholesterol-containing foods which are subjected to common processing conditions such as spray-drying and deep-fat frying, and during prolonged storage. COPS have been identified in a variety of foods including raw, cooked, and dehydrated meats (Sander et al., 1989), dairy products (Cleveland and Harris, 1987), fish products (Ohshima et al., 1993; Li, et al., 1994) and egg products (Naber and Biggert, 1985; Addis, 1986). Factors such as time of storage and exposure to light, air, or heating temperature during the processing, packaging, and storage of foods can have significant effects on the production of COPs in foods.

1.6.1. Egg Products

Dehydration provides ready-to-cook foods such as cake and cookie mixes, casserole dishes, dessert mixes, etc (Stewart, 1973). The advantage of processed foods is the long food storage time, making many varieties of foods available throughout the year. This industry has greatly expanded in recent decades because of the light weight, stability, and excellent functional properties, such as binding, thickening, emulsifying, texturizing, and moistening of dried egg powder. It is used in almost all baked products (Johnson, 1974). The food spray-drier depicted in Figure 1-4., is used for the production of most dried egg and milk products, which are in powder form (Stewart, 1973). During the process, egg yolk is atomized and dried by hot air directly from a gas burner, or indirectly from the surface of steam coils. The temperatures applied are measured at the inlet and outlet after drying the product. About 60 % of the yolk solids are lipids, approximately

4% of which is cholesterol (Tsai and Hudson, 1984). A large surface area is created by atomization, and together with the high operating temperature (about 60°C). The reactions between lipids and molecular oxygen is accelerated, thus yielding cholesterol oxides in dried egg powder (Tsai and Hudson, 1984).

Nourooz-Zadeh and Appelqvist (1987) determined COPs in fresh eggs and dehydrated egg products. No COPs could be detected in fresh eggs. Similarly, spray dried egg yolk powder contained only traces of COPs when fresh, or stored for 2 months at 4°C, but, prolonged storage gave extracts containing COPs. The levels of COPs were higher if stored at ambient temperature than stored in 4° to 8°C. However, Emanuel et al (1991) found COPs in fresh spray dried egg powder (Table 1-1). Tsai and Hudson (1985) found that spray-dried eggs contained relatively high levels of COPs (Table 1-2). Prolonged storage increased the COPs content in dried egg products.

Missler et al. (1985) separated and identified several cholesterol oxides, such as α- and β-epoxides and 7-ketocholesterol from powdered scrambled egg mix, in a standard military food which had been stored for 5 years. Cholesterol oxidation product concentrations found in dried egg mix heated by two different methods, direct and indirect ranged from 5.1-50.0 ug/g and 1.4-21.5 ug/g, respectively, (Table 1-3). The direct air heating method significantly increased production of COPs. The epoxides can undergo hydrolysis and produce cholestanetriol, a potent atherogenic compound in experimental animals. Keeping the samples cool, under nitrogen, and in the absence of light, helped to reduce further oxidation.

1. 6. 2. Meat Products

1. Beef Tallow

Park and Addis (1986a) investigated COPs in heated beef tallow at various frying temperatures, 135°C, 150°C, 165°C, and 180°C, by continuous heating. They identified at least four COPs, 7α-OH-cholesterol, 7β-OH-cholesterol, 7-ketocholesterol and α-epoxide. 7-ketocholesterol was the predominant species formed (Park and Addis, 1986a, b). Bascoul et al. (1986) also detected relatively large quantities of COPs (100-300 ppm) in beef tallow used for deep-fat frying.

2. Processed Meats

Processed meat products also contain varying amounts of COPs as shown in Table 1-4 (Higley, 1986). Freeze-dried pork, stored in contact with air at 22°C for ca. 3 yrs, contained 7α- and 7β-OH-cholesterol, 7-ketocholesterol, α- and β-epoxides and cholestane-triol (Park and Addis, 1987).

1.6.3. Dairy Products

1. Milk

Nourooz-Zadeh and Appelqvist (1988) published the first quantitative data on COPs in milk powder products. They analyzed powdered samples of cream, whole milk and skim milk from different manufacturing plants in Sweden, prepared under various conditions. Freshly-made roller-dried cream, whole milk and skim milk powder contained no COPs for low and medium heat powders. However, fresh spray-dried whole and skim milk powders from the high heat category contained quantifiable amounts of α- and β-

epoxides, 7-ketocholesterol and 7-OH-cholesterol. Spray-dried whole milk had increased levels of COPs at room temperature after 1 year (Nourooz-Zadeh and Appelqvist, 1988), which are shown in Table 1-5 and Table 1-6. The same authors also investigated the presence of cholesterol oxides in fresh dried milk powder from New Zealand (Table 1-7).

2. Butter

Because of its high lipid content, butter is susceptible to deterioration resulting in flavor and /or color alteration (Luby et al., 1986a). Oxidation is one of the causes of these alterations and it occurs especially when the butter is inadequately protected. The severity of deterioration depends upon factors such as the common fluorescent light, exposure time, wavelength of light, temperature of butter, distance of butter from light source, and salt and β-carotene contents of butter. Luby et al. (1986b) reported no COPs in the original butter (day 0), but with increasing exposure, 7-OH-cholesterol increased linearly with higher concentrations at the surface layer of the butter than throughout the entire block. However, the higher detectable amount of COPs at the surface demonstrates that photoxidation is the major cause of COPs formation in butter. Packaging material as well as the light source affect the oxidation of cholesterol in butter (Luby et al., 1986b). Cholesterol oxidation rate was approximately parallel to light transmission characteristics of the packaging materials, and only aluminum foil prevented peroxide formation and oxidation of cholesterol after 15 days of exposure.

Csiky (1982) identified COPs in butter and in heated butter. Butter (1.0 g) was heated for 5 and 10 min in an open glass vessel kept at 180°C. The amounts of various cholesterol oxides found ranged from 2.6 ug/g in unheated butter to 52.2 ug/g in butter

heated for 10 min (Table 1-8).

3. Cheese

Finocchiaro et al. (1984) detected cholesterol oxides in aged, grated Italian cheeses such as Parmesan and Romano. Samples of both cheeses packaged in clear glass containers had the highest amounts of cholesterol oxides (Table 1-9), while those packaged in cardboard contained lower amounts. Photoxidation may be responsible for the greater amounts of cholesterol oxides in clear glass containers.

1. 6. 4. Fish Products

Fish products including salted and dried, boiled, dried and smoked products, and commercial fish oil, range from 8.3 ppm to 188.0 ppm with 7β-OH-cholesterol and 7-ketocholesterol being the most prominent oxidation decomposition products of cholesterol (Ohshima et al., 1993; Li et al., 1994). In general, foods based on marine resources have a wide variety of constituent fatty acids having carbon chains from 14 to 22 carbon units in length, and with up to 6 ethylenic bonds. Long chain polyunsaturated fatty acids (LCPUFAs) (20:5n3 and 22:6n3) are high in marine foods. These PUFAs are susceptible to autoxidation and enhanced cholesterol oxidation (Ohshima et al., 1993).

1. 6. 5. Various Foods

Cholesterol oxidation products were also determined in various foods of animal origin by Fischer et al. (1985). The major oxidation products were 7-OH-cholesterol, α- and β- epoxides. Foods obtained from fast-food restaurants such as cooked hamburger,

french fried potatoes (fried in beef tallow), deep-fat-fried chicken, as well as pancake mix, fresh bovine brain and liver, liver sausage and beef jerky were analyzed by Park and Addis (1985a). Pancake mix, french fries and organ product "concentrates" contained from 1 to 70 ppm of 7-keto- and 7-OH cholesterols, but none could be detected in raw beef, fried chicken, cooked hamburger, beef jerky or liver sausage (Park and Addis, 1985a; Hubbard et al., 1989). Lee et al. (1985) detected α- and β-epoxides, and 7-OH-cholesterol in samples of french fries from five different fast-food restaurants. The quantity of COPs detected ranged from undetectable to 81 ug/g of lipid as shown in Table 1-10. The presence of COPs in french fries is possible, since fast-food restaurants sometimes use beef tallow for frying at high temperature in the presence of light and oxygen and it is used over a relatively long period of time.

1. 7. Control of Cholesterol Oxidation in Foods

1. 7. 1. Use of Antioxidants

Several natural and synthetic compounds with antioxidant activity have been evaluated and employed in model systems, meat and meat products to control lipid peroxidation. Antioxidants work in two ways (Peng, 1985): 1) Antioxidants function as free radical terminators by donating a hydrogen atom to the lipid free radical thereby stopping the chain reaction. Some examples of these compounds are butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylhydroquinone (TBHQ) and propyl gallate which are synthetic, and tocopherol and carotene which are natural

compounds. 2) Antioxidants are metal complexing agents, or chelators, and function as free radical preventers by controlling the production of free radicals during ionization, Examples of these types of compounds are EDTA, citric acid, phosphates and ascorbic acid. In addition to the health concerns about the presence of additives in food, the affectivity of such antioxidants is variable. Imaida et al. (1983) reported BHA and BHT may promote carcinogenesis in rats. Rankin and Pike (1993) found that BHA had no antioxidant properties for cholesterol, and tocopherols can delay cholesterol oxidation. Maerker and Unruh (1986) also reported that BHT did not inhibit cholesterol autoxidation. In contrast, Sagers (1991) demonstrated that BHA, BHT, propyl gallate, TBHQ and α-tocopherol inhibited cholesterol autoxidation in an aqueous model system.

There have been many reports on the protective effect of dietary vitamin E on lipid stability in various muscle foods. For example, supplementation of veal calves with vitamin E improved oxidative stability of rendered fat (Ellis et al., 1974), even in animals whose diets had been supplemented with high levels of linoleic acid. Shorland et al. (1981) observed a protective effect of vitamin E supplementation on lipid stability in longissimus dorsi tissue. Faustman et al. (1989) theorized that the improved lipid and colour stability of muscle from Holstein steers fed diets supplemented with 370 IU vitamin E/day was the result of the incorporation of vitamin E into membranes. Several recent studies have focused on the effects of vitamin E supplementation on the incorporation of α-tocopherol into membranes and the resultant oxidative stability of membranes and muscles in different species. Monahan et al. (1992) showed the incorporation of α-tocopherol into mitochondria and microsome and a resultant

improvement in lipid stability as a consequence of supplementing pig diets with 200 mg α-tocopherol acetate/kg feed. Asghar et al. (1989) reported increased concentrations of α-tocopherol in porcine tissues with increasing dietary levels of vitamin E, and a resultant improvement in muscle and membranal lipid stability. Similar trends were found in poultry (Asghar et al., 1990). The α-, β-, γ- and δ-tocopherols differ in the degree of methylation of the dihydrochromanol ring. Their antioxidant activities depend very much on the food to which they are added, and the presence of heavy metals and various synergists (Chan, 1987). At high concentrations and in the presence of iron and copper salts, tocopherols may act as pro-oxidants. Satisfactory antioxidant activity is usually achieved only when they are used in combination with synergists such as ascorbic acid, citric acid and some amino acids or with various chelating agents. The antioxidant activities of tocopherols range in the following order: δ-tocopherol(most effective) > γ-tocopherol > β-tocopherol > α-tocopherol (least effective) (Pokorny, 1991). Natural tocopherols usually consist of mixtures of α-, β-, γ-, and δ-tocopherols. They are readily available from the sludges formed during the deodorization of refined vegetable oils. Tocopherol preparations of sludges have better antioxidant activity than pure α-tocopherol because of their content of γ- and δ-tocopherols. Natural γ-tocopherol efficiently stabilizes stored edible oils. Tocopherol oxidation products also have some antioxidant activity, and the structurally related α-, β-, γ- and δ-tocotrienols, which are found in palm oil, have antioxidant activities similar to those of the respective tocopherols. Consumers prefer natural compounds such as vitamin E (α-tocopherol) for preventing oxidation (Pokorny, 1991). Vitamin E can be indirectly or directly incorporated in food to prevent

lipid autoxidation.

1.7.2. Environmental factors

The conditions of processing, packaging, and storage of foods have a profound effect on the production of cholesterol oxides. Therefore, attention should be given to the method of packaging and storage, as well as packaging material, time of storage, exposure to the light, air, heating temperatures, and other variables which increase the content of cholesterol oxides in foods.

1. 8. Analysis Methods for COPs

Cholesterol oxides are present in foods in rather low concentration, and their isolation and determination have presented challenging analytical problems. A number of methods have been developed for the quantitative determination of cholesterol oxides in various food products exposed to heat and air during manufacturing and / or long term storage, but no standard method as yet exists which combines the essential qualities of accuracy, precision, and selectivity with that of speed (Nourooz-Zadeh, 1990). Primary attention has been focused on the isomeric 5, 6-epoxycholesterols (Tsai and Hudson, 1984) or the epimeric 7-hydroxycholesterols and 7-ketocholesterol (Herian and Lee, 1985; Park and Addis, 1985a).

All methods for analysis of COPs from tissues and foods require concentration of the more polar COPs by removal of extraneous lipids and the bulk of cholesterol. Previous workers in the field frequently have employed saponification of the extracted

lipids with hot alkali as an important part of the enrichment procedure (Naber and Biggert, 1985; Finocchiaro and Richardson, 1984). This step frees the bulk of the lipids from the sterol residue and also converts esterified cholesterol to the free sterol. Nevertheless, some important cholesterol oxides, notably 7-ketocholesterol, have long been known to be unstable in the presence of hot and aqueous alkali (Finocchiaro and Richardson, 1983), even with mild saponification (Van de Bovenkamp, 1988).

Researchers have employed an isolation and prefractionation step using silica column chromatography (Tsai et al. 1980; Park and Addis, 1985a; Van de Bovenkamp et al. 1988). Park and Adiss (1985a) reported that hexane-ethyl acetate (9:1) was the optimum solvent mixture to achieve the best separation of cholesterol oxides from cholesterol and triglycerides, and acetone eluted all sterol oxides, leaving phospholipids on the column.

Thin-layer-chromatography (TLC) greatly simplified the detection and study of cholesterol oxidation products in the early 1960's. Smith and coworkers (1967) showed that TLC R_f values and colors of spots after spraying can be combined to provide a lead to the identity of components of a mixture. The usual procedure is to develop the mixture of oxides with solvent pairs such as heptane:ethyl acetate (1:1), and then to spray the developed TLC plate with 50% sulphuric acid followed by heating at 110-120°C. The brilliant colors that develop range from magenta for cholesterol, blues for some of the diols and epoxides, and yellow for triol. Some of the colors are fairly fleeting, and it is customary to char the plates completely at higher temperatures (Mearker, 1987). TLC is also another choice for the fractionation of lipids into classes and was used for the

determination of cholesterol α-oxide from human serum (Gray et al., 1971). However, TLC is also laborious and perhaps inaccurate for the purpose of quantification due to the scraping, elution and transferring procedures involved, and it is difficult to collect many kinds of cholesterol oxidation products by one TLC irrigation. TLC allows exposure of the samples to potential oxidation as a result of its large surface area, and also suffers from low loading capacity. Therefore, one is unlikely to recover ug quantities of cholesterol oxides from mg amounts of cognate lipids. The means of detection ultimately limits the usefulness of TLC for quantitation of cholesterol oxidation products (Smith et al., 1967; Smith and Hill, 1972; Park and Addis, 1985a).

High performance liquid chromatography (HPLC) is a popular method for steroid separation. Semipreparation HPLC is useful in separating cholesterol, triglycerides and other lipids from the cholesterol oxides and thereby enriches the oxide fraction when saponification is avoided (Maerker and Unruh, 1986). HPLC has been used by a number of workers to analyze cholesterol oxidation products (Missler et al., 1985; Tsai and Hudson, 1981). Both reverse phase and normal phase HPLC systems have been used. The latter has been more successful, and good separations have been obtained with hexane/isopropanol combinations in which the hexane phase comprised 90-99%. The procedure is highly effective for separation of diols and hydroperoxides, but triol retention time is excessive, and this compound is often not determined in adsorption HPLC (Maerker, 1987). Tsai and Hudson (1981) studied the elution profile of various cholesterol oxidation products on a normal-phase silica column with various mobile phases. Good resolution of the isomeric 5, 6-epoxides has been reported (Tsai et al., 1980). However,

certain cholesterol oxides that are less polar than cholesterol are lost in this procedure and some have problems with separation from large amounts of interfering compounds such as lipids from egg yolk, butter, and tallow (Maerker, 1987). A published HPLC method used silica gel column chromatography for preliminary purification of samples (Park and Addis, 1985a).

Gas chromatography (GC) has been used in the analysis of cholesterol oxidation products for some time, but resolution on packed columns has not been satisfactory (Teng et al., 1973). The recent development of capillary GC has permitted better separations of the principal oxidation products (Park and Addis, 1985b). However, pretreatment of samples is necessary before chromatography. Free sterols have been reported to show significant thermal decomposition and poor resolutions during GC separation (Nawar et al., 1991). Several researchers (Missler et al., 1985; Park and Addis, 1985b) have prepared trimethylsilyl ether (TMS) derivatives of the cholesterol oxides to alleviate these problems.

Mass spectrometry is very important for effective analyses, as identity of analyte, sensitivity for detection, and linearity for estimation are all well served. Complete spectra obtained by electron impact or by chemical ionization using methane or ammonia as reagent gases coupled with chromatographic data generally serve to identify each cholesterol oxide satisfactorily (Lin, 1980; Lin and Smith, 1984).

Selected ion mass spectrometric measurements are also emerging as very useful for detecting, confirming identity, and estimating amounts of cholesterol oxides in complex mixtures. Cholesterol oxide trimethylsilyl ethers are best used. Cholesterol oxide

levels in rat liver microsomes have been measured in that manner (Sanghvi et al., 1981).

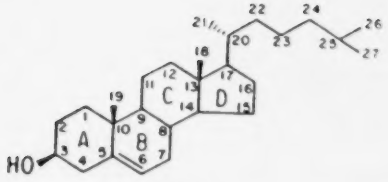
These methods provide means to analyze cholesterol oxides with specificity and sensitivity essential for serious work. However, these means fail to guarantee adequate analysis of cholesterol oxidation products and use of an appropriate bioassay is recommended (Lacko, 1980). Moreover, whatever analysis method is used, there remains the issue of generation of artifacts during the process (Smith, 1987).

1. 9. Objectives of This Thesis

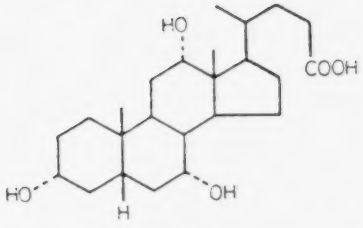
The influence of dietary cholesterol on plasma and lipoprotein cholesterol level and coronary heart disease (CHD) risk has been studied extensively over the last four decades. This relationship has been examined in experimental animals, clinical studies and observational epidemiological studies ranging from multinational comparisons to much smaller groups with distinct eating habits. (Wissler and Vesselinovitch, 1987: Stamler and Shekelle, 1988; McGill et al., 1979; Grundy and Denke, 1990). In addition, cholesterol is an important precursor to a variety of key biological substances. It is essential for growth, development, and normal physiological functions of the body. However, the formation of cholesterol oxides proceeds via free radical oxidation similar to autoxidation of polyunsaturated fatty acids, acting upon elevated cholesterol levels resulting in the accumulation of these potentially cytotoxic and atherogenic products (Howard, 1991). PUFA may enhanced cholesterol oxidation. Foods high in cholesterol when exposed to air, light, and heating, may be important sources of dietary cholesterol oxides associated with atherosclerosis. Scientific study of cholesterol autoxdation in food has become a

critical research need. Therefore, the objectives of this thesis: 1) study the effect of storage time, cooking temperature and tocopherol on cholesterol oxidation in different food oils. 2) investigate the effects of varying fatty acid composition and tocopherols content of dried egg yolk powders, by dietary supplementation of laying hens, on the subsequent susceptibility of powders to cholesterol oxide formation during storage at room temperature and with cooking (heating). 3) determine the effects of dietary oils and tocopherol supplementation on cholesterol oxidation in chicken meat during storage.

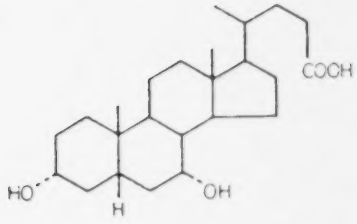
Figure 1-1. Structure of Cholesterol and Related Compounds



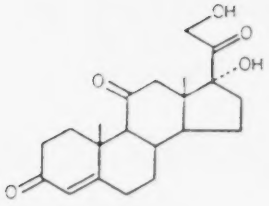
Cholesterol



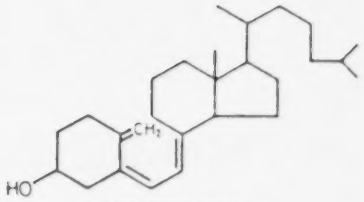
Cholic acid



Chenodeoxycholic acid

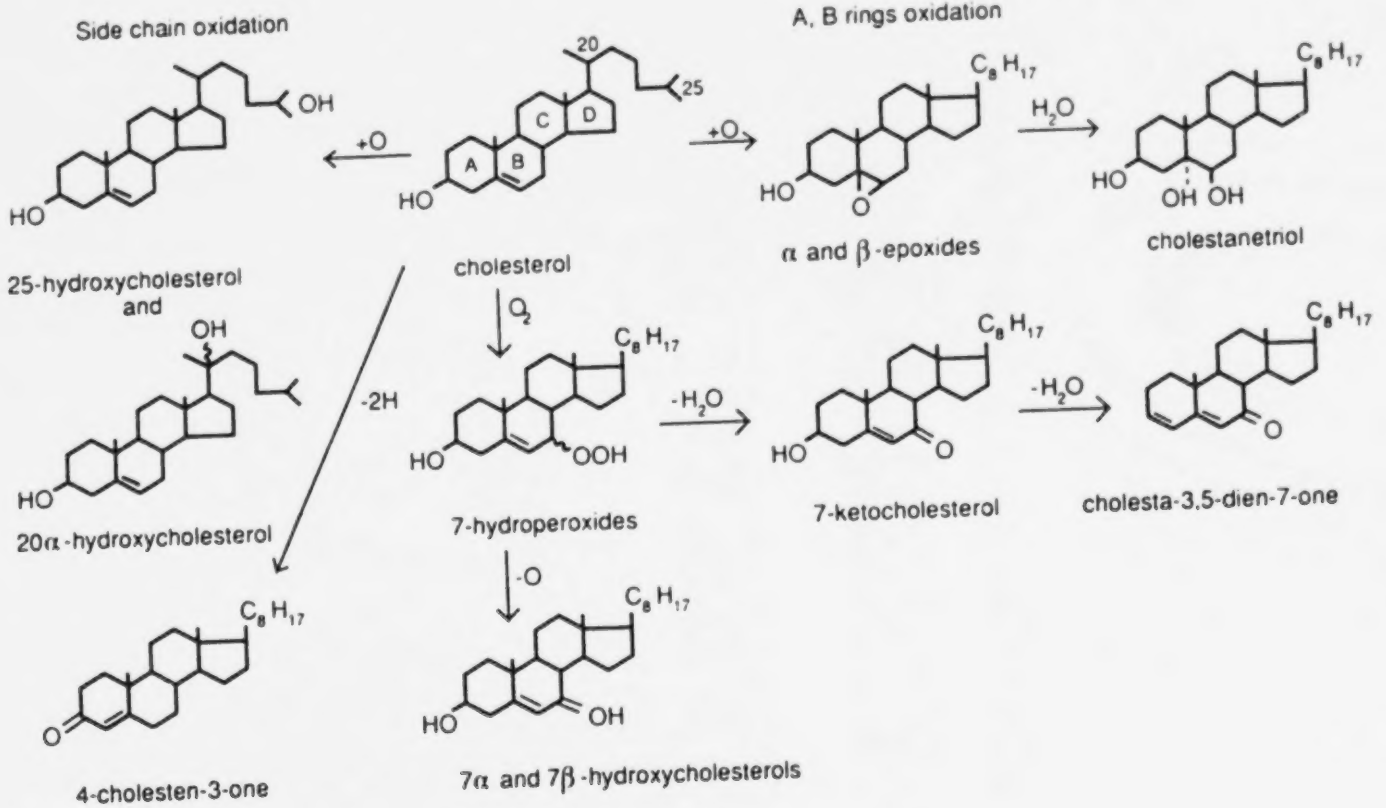


Cortisone



Cholecalciferol
(9,10-Seco-5,7,10(19)-
cholestatrien-3β-ol)

Figure 1-2. Mechanism and Major Products of Cholesterol Oxidation
Source: Peng, S. K. and Taylor, C. B. World Rev. Nutr. Diet 1984. 44: 117-154. Reproduced from Hubbard et al., Prog. Food Nutr. Sci. 1989. 13: 17-44


Side chain oxidation
A, B rings oxidation
+O
+O
20
25
C
D
A
B
HO
OH
25-hydroxycholesterol
and
OH
HO
20α-hydroxycholesterol
cholesterol
C_8H_{17}
HO
O
α and β-epoxides
H_2O
C_8H_{17}
HO
OH OH
cholestanetriol
O_2
C_8H_{17}
HO
OOH
7-hydroperoxides
-2H
-H_2O
C_8H_{17}
HO
O
7-ketocholesterol
-H_2O
C_8H_{17}
O
cholesta-3,5-dien-7-one
-O
C_8H_{17}
O
4-cholesten-3-one
C_8H_{17}
HO
OH
7α and 7β-hydroxycholesterols

Initiation: $RH + O_2 \dashrightarrow ROO. + H.$

Propagation: $R. + O_2 \dashrightarrow ROO.$

$ROO. + RH \dashrightarrow ROOH + R.$

Termination: $ROO. + R. \dashrightarrow ROOR$

$R. + R. \dashrightarrow R - R$

$ROO. + ROO. \dashrightarrow ROOR + O_2$

Figure 1-3. Mechanism of Lipid Oxidation

Figure 1-4. Food Spray Drier
Source: Stewart, G. F. and Maynard, A. A. New York: Academic Press, 1973. 164-171.

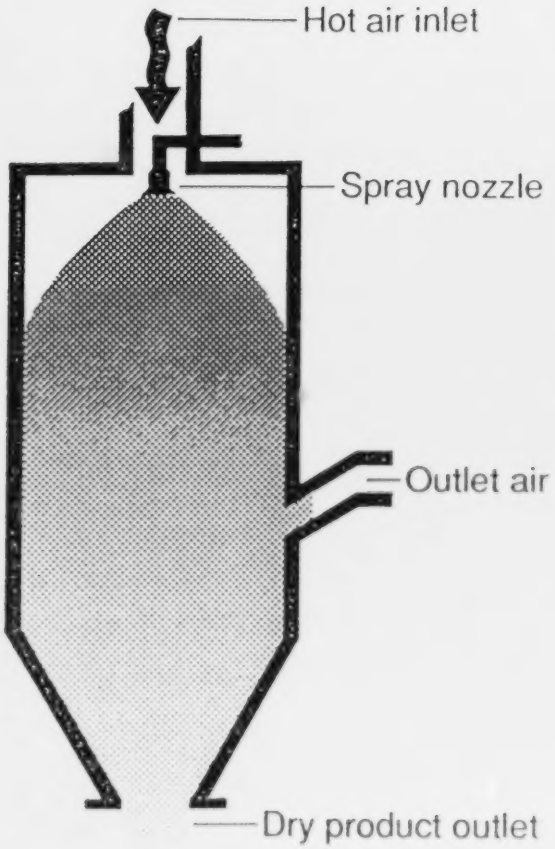

Hot air inlet
Spray nozzle
Outlet air
Dry product outlet

Table 1-1. Cholesterol Oxidation Products (COPs) in Fresh Eggs and Fresh Powdered Eggs

	Egg products (ppm)	
COPs	Powder	Fresh
Cholestane-β-epoxide	50	2
Cholestane-α-epoxide	90	0
7β-hydroxycholesterol	60	0
7-ketocholesterol	30	2

Adapted from the work of Emanuel et al., 1991. J. Food Sci. 56: 843-847.

Table 1-2. Effect of Storage Time on Formation of Epoxides (ppm) in Dried Egg Products

Sample	Egg Products	Original	9 Months	Increase (%)
1	Whole egg + sucrose	236	258	9
2	Plain yolk	18	70	289
3	Plain whole	36	166	361
4	Whole egg + CSS*	ND+	110	NA#

* Corn syrup solids.
+ None detected.
# Data not available.
Adapted from the work of Tsai and Hudson, J. Food Sci. 1985, 50:229-237.

Table 1-3. Effect of Type of Heat Treatment on Oxysterols Formation in Spray-Dried Egg Mix

COPs	Indirect (ug/g)	Direct (ug/g)	Increase (%)
7α-OH	1.8	7.0	289
7β-OH	1.5	18.5	1133
α-Epoxide	21.5	50.0	132
β-Epoxide	1.9	37.4	1868
25-OH	1.4	5.1	264
7-keto	2.0	37.0	1750
Triol	11.6	13.0	12

Adapted from the work of Missler et al., J. Food Sci. 1985. 50: 595-598, 646.

Table 1-4. Cholesterol Oxides (ppm/food sample) in Processed Meats

Processed meat	25-OH	7α-OH	7β-OH	Triol
Cooked Bratwurst	TR*	820.0	TR	1335.0
Beef franks	TR	98.5	34.0	TR
Chicken roll	TR	TR	TR	TR
Cooked hamburger	TR	72.0	NA+	NA
Cooked lean bacon	TR	TR	NA	TR
Raw hamburger	TR	TR	36.0	1298.0
Turkey bologna	NA	NA	NA	86.0

*Trace
+ Data not available.
Adapted from the work of Higley et al. 1986. Meat Sci. 16: 175-188.

Table 1-5. Oxysterols in Fresh Milk Powder Products From Sweden Treated by Different Temperature*

Treatment	Products	α-Epoxide	β-Epoxide	7-keto	7α-OH	7β-OH
Low heat	a+	ND#	ND	ND	ND	ND
Low or Medium	Skim milk powder	ND	ND	ND	ND	ND
High heat	Whole milk powder	0.6** (16)##	0.2 (5)	TR++	1.4 (38)	0.6 (16)
	Skim milk powder	2.6 (1.8)	0.9 (0.6)	TR	2.0 (1.4)	2.1 (1.5)

* Temperatures applied in the concentration of milk.
+ Cream powder, whole and skim milk powder.
# None detected.
** Parts per million, mean of duplicate analysis.
++ Traces
Number in parenthesis representd amount of oxysterols (mg) per 100 g of food sample.
Adapted from the work of Nourooz-Zadeh and Appelqvist, J. Food Sci. 1988. 53:74-87.

Table 1-6. Cholesterol Oxides in Stored Dried Milk Powders from Sweden

Products	Months	α-Epoxide	β-Epoxide	7-keto	7α-OH	7β-OH
Whole milk (n=3)	12	1.55 ± 1.34*	2.15 ± 1.48	7.15 ± 2.9	0.4 ± 0.14	1.1 ± 0.5
		42 ± 36+	58 ± 40	193 ± 78	11 ± 4	30 ± 13
Skim milk (n=14)	13-37	1.95 ± 0.86	5.4 ± 2.9	11.8 ± 4.5	10.7 ± 3.1	15.8 ± 4
		1.3 ± 0.6	3.7 ± 2	8.2 ± 3	7.4 ± 2	11 ± 3

* Parts per million, mean ± SD of composite means.
+ Mean ± SD of oxysterol (mg) in 100 g of food sample.
Adapted from the work of Nourooz-Zadeh and Appelqvist, J. Food Sci. 1988. 53:74-87.

Table 1-7. Cholesterol Oxides in Fresh Dried Milk Powders from New Zealand

Products	7α-OH	7β-OH	7-keto	α-epoxide	β-epoxide
Whole milk	ND*	ND	0.4	0.6*	0.3
			(11)#	(16)	(8)
Skim milk	1.0	2.3	2.5	0.8	0.9
	(0.7)	(1.6)	(1.8)	(0.6)	(0.6)

* Parts per million, means of duplicate analysis.
+None detected
Approximate amount of oxides (mg) in 100 g of food sample.
Adapted from the work of Nourooz-Zadeh and Appelqvist, J. Food Sci. 1988. 53: 74-87.

Table 1-8. Cholesterol Oxides (ug/g of butter) in Unheated and Heated Butter

Butter	7α-OH	7β-OH	7-keto	25-OH
Unheated	NA*	NA	NA	2.6
Heated+	19.6	8.4	5.2	34.0
Heated#	52.2	19.7	11.2	NA

* Data not available.
+ Heated For 5 min at 180 C.
# Heated for 10 min at 180 C.
Adapted from the work of Csiky, J. Chrom. 1982. 241:381-389.

Table 1-9. COPs (ug/g of cheese)* in Grated Aged Italian Cheeses

Cheese	Packaging material	Epoxides	7α-OH	7β-OH	Triol
Parmesan Brand A	Clear glass	32 ± 2.9	6 ± 0.6	6 ± 0.6	2 ± 0.6
Parmesan Brand A	Cardboard box	9 ± 1.2	3 ± 1.2	3 ± 1.2	TR+
Parmesan Brand D	Cardboard box	6 ± 1.2	TR	TR	TR
Romano Brand A	Clear glass	16 ± 1.6	3 ± 0.6	3 ± 0.6	TR
Romano Brand A	Cardbord box	ND#	ND	ND	ND

7α-OH = 7α-Hydroxycholesterol; 7β-OH = 7β-Hydroxycholesterol.
* Mean ± SD.
+ Traces.
# None detected.
Adapted from the work of Finocchiaro et al., J. Am. Oil Chem. Soc. 1984. 61:877-883.

Table 1-10. Cholesterol Oxides (ug/g of lipid) in French Fries

Sample*	α-Epoxide	β-Epoxide	7α-OH	7β-OH
5	9.3±6.7	13.1±9.3	31.1±25.2	7.4±6.4

Mean ±SD
* Obtained from 5 different fast-food restaurants.
Adapted from the work of Lee et al., 1985. 48: 158-161.

1. 10. Reference

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Chapter 2. [1]Effects of Storage, Heating and α-Tocopherol Supplementation on Cholesterol Oxide Formation in Different Food Oils

2. 1. Introduction

Cholesterol can undergo autoxidation in air to form cholesterol oxide products (COPs) known as powerful atherogenic agents both in vivo and in vitro (Addis, 1986; Hubbard et al., 1989). Steady consumption of clarified butter, which included a high level of cholesterol oxides, by Indian immigrants in the United Kingdom was assumed to explain the higher-than-expected mortality from atherosclerosis (Jacobson, 1987). The production of cholesterol oxides in many types of food and foodstuffs has been reported, including heated fats and oils (Park and Addis, 1986a,b; Zhang et al., 1990; Yan and White, 1990), deep-fried foods (Bascoul et al., 1986), meat products (Park and Addis, 1987; Zubillaga and Maerker, 1991), milk and egg products (Naber and Biggert, 1985; Nourooz-Zadeh et al., 1988).

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Cholesterol and lipid autoxidation is a free radical-mediated process which occurs in foods and especially in cooked foods. Deep fat frying is a common method of food preparation in restaurants and in processing plants. During frying, the heating medium may experience abusive conditions due to repeated exposure to oxygen at elevated temperatures (Park and Addis, 1986a,b). The thermal oxidation and polymerization of fatty acids, including toxicological implications and loss of nutritional value have been reported (Kaunitz, 1967; Chang et al., 1978; Thompson and Aust, 1983). However, the apparent formation of cholesterol oxides was not favoured at elevated temperatures. Heating cholesterol at very high temperatures, such as 200-300°C, may have caused thermal degradation of cholesterol rather than oxidation (Park and Addis, 1986a). It has also been reported that cholesterol was oxidized in conjunction with autoxidation of coexisting triacyglycerols that contained polyunsaturated fatty acids (Li et al., 1994). Polyunsaturated fatty acids (PUFAs) are high in fish and seafood (20:5n3, 22:5n3 and 22:6n3), leaf vegetables and pods (18:3n3) and some oil seeds (18:3n3) (Leaf and Weber, 1988). These PUFAs are very susceptible to peroxidation, even under mild ambient conditions, and are easily incorporated into the chain reaction of lipid peroxidation to yield free radicals and peroxy radicals (Hsieh and Kinsella, 1986). It is logical, therefore, to suspect that the oxidation of cholesterol might be accelerated in the presence of peroxidized lipids. Ohshima et al. (1993) found that a continuous increase in the amount of cholesterol oxides was accompanied by a remarked concurrent decrease in polyunsaturated fatty acid residues. Therefore, compounds which inhibit unsaturated fatty acid oxidation may reasonably be expected to inhibit cholesterol oxidation.

There have been many reports on the antioxidant effect of tocopherols in foods (Dougherty, Jr. 1988). Vitamin E functions as a lipid-soluble antioxidant in cell membranes (Linder, 1985), and the most potent form of vitamin E, α-tocopherol is capable of quenching free radicals and thus, protects phospholipids and cholesterol against oxidation, and subsequent breakdown to potentially harmful, chemically reactive products (Faustman, 1989). Tocopherols are major antioxidants in vegetable oils (Sherwin, 1978) and show a synergistic effect with phospholipids and ascorbic acid for enhancing oxidative stabilities of oils (Hudson and Mahgoub, 1981). Dietary vitamin E (α-tocopherol) supplementation improves the oxidative stability of muscle from chicken (Asghar et al, 1990) and pork (Monahan, 1992). Considering the importance of PUFAs and tocopherols of lipid oxidation, the objective of this study was to determine the effect of fatty acid composition and α-tocopherol on the cholesterol oxidation in food oils during storage and heating.

2. 2. Materials & Methods

Reagents

Cholesterol and cholesterol oxide standards (>99% purity), including 7α-hydroxycholesterol, 7β-hydroxycholesterol, α-epoxide, β-epoxide, cholestanetriol, 7-ketocholesterol and 6-ketocholestanol, were purchased from Sigma Chemical Co. (St Louis, MO) and Steraloids Inc. (Bayonne, New Hampshire). Tocopherol isomer standards were purchased from Sigma Chemical Co. (St Louis, MO). All standards were used as purchased. Celite 545 and calcium phosphate ($CaHPO_4 \cdot 2H_2O$) were purchased from

Fisher Scientific Co. (Malvern PA) and silicic acid (100 mash) from Aldrich Chemical Co. (Milwaukee, WI). Acetonitrile and methanol were HPLC grade from BDH Inc. (Toronto, Ontario), and all other solvents used were glass distilled.

Experiment Design

The four different oils, fish, flax, sunflower and palm oils, were purchased from commercial food oil producers. Duplicate 40 g samples of each oil were weighed into a 50 ml culture tube and 400 mg of pure cholesterol (checked for cholesterol oxides by thin layer chromatography) was added to each tube and 16 mg of pure α-tocopherol was added to one of the duplicate tubes (400 ppm in the oils), then samples were well mixed. Samples were divided into three equal aliquots for storage study. Samples stored at room temperature and open to air, were mixed daily to ensure uniformity of light (normal daylight cycle) and air access. One sample was taken from each tube for cholesterol oxides, two samples from each tube for tocopherols and fatty acids and analyzed at 0, 15 and 35 days during the period of storage. For heating treatment experiments, sample preparation was the same as in storage treatment studies. Samples were heated in an air convection oven at 110°C for 22 hrs and then analyzed. One sample for cholesterol oxides from each tube and two samples for fatty acids and tocopherols from each tube were analyzed before and after heating.

Fatty Acid Composition Analysis

Fatty acid methyl esters were prepared from oil samples by transesterification with

BF3-Methanol (Morrison and Smith, 1964). Analyses were performed on a Varian 3600 gas chromatography (GC) (Varian Associates Inc., Walnut Creek, CA 94598) equipped with an 8200 autosampler, an on-column injector and a flame ionization detector (FID). A DB-23 (J and W Scientific, Folsom CA) 30m x 0.25mm i.d. x 0.25um film thickness fused silica capillary column was used to separate fatty acid methyl esters. Column temperature was programmed from 70°C for 0.5 min to 180°C at 30°C/min and held there for 10 min then increased to 230°C at 5°C/min and held at the final temperature for 3 min. Injector temperature was programmed from 80°C to 230°C at 150°C/min, and held at 230°C for 17 min. Liquid CO_2 was used to cool the injector. Helium carrier at gas head pressure was set at 20 psi and a flow rate of 30 ml/min as a make-up gas. Peak areas were integrated using a Shimadzu EZChrom chromatography data system (Shimadzu Scientific Instruments Inc., Columbia MD).

Tocopherol Assay

A weighed amount (1 g) of oil in duplicate was placed into a 10 ml tissue culture tube. Samples were diluted with 1ml hexane and mixed well. Twenty ul of mixture was placed into a 2ml autosampler vial, and 200 ul of the internal standard (rac-5,7-dimethyltocol, 10ug/ml in methanol) was added. Twenty five µl volume was injected onto the HPLC column. A Varian 5000 liquid chromatography system with a Shimadzu Sil-9A model autosampler was used to determine α, γ and δ-tocopherol concentrations in the oils. Separation was achieved on a 3um Supelcosil, RPLC-18 column, 150 x 4.6 mm i.d. (Supelco Canada Ltd., Oakville, Ont.), with a guard column (5cm, 20-40 um LC-

18 packing) and a mobile phase of methanol:acetonitrile (50:50 v/v) at a 1.5ml/min flow rate. Tocopherol isomers were detected with a Shimadzu RF-535 fluorescence detector at an excitation wavelength of 295 nm and an emission wavelength of 330 nm. A Shimadzu EZChrom chromatography data system (Shimadzu Scientific Instruments Inc., Columbia MD) was used to integrate peak areas.

Cholesterol Oxidation Products (COPs) Analyses

1. Sample preparation

The column chromatography method for isolation of cholesterol oxides developed by Zubillaga et al (1991) and Park and Addis (1985) was used with some modifications. A homogenous mixture containing silicic acid (100 mesh), Celite 545 (Fisher Scientific) and $CaHPO_4 \bullet 2H_2O$ (10:9:1 wt/wt/wt) was made into a slurry with chloroform, and packed into a glass column (12mm i.d. x 30cm) to a height of 15 cm. The column was washed with 10 ml hexane:ethyl acetate (9:1 v/v, solvent-I) before samples were applied. Oil samples, 0.3g, dissolved in 5 ml solvent-1 and 10ug of 6-ketocholestanol, as an internal standard, were applied to the column. Neutral lipids and cholesterol were eluted by passing 50 ml solvent-1 and then 35 ml hexane:ethyl acetate (8:2 v/v, solvent-II) through the column. Elution of cholesterol oxides was accomplished with 40 ml of acetone:ethyl acetate:methanol (50:50:5 v/v/v, solvent-III) at approximately 1ml/min flow rate.

The final elute was dried under nitrogen, and the cholesterol oxidation products were converted to TMS derivatives with 200 ul pyridine and 100 ul Sylon BFT (Supelco Inc., Bellefonte, PA) at 80°C for 1 h.

2. Gas chromatography and Mass spectrometry

Analysis of cholesterol oxides was performed on a Varian 3400 GC with an on column injector and a FID detector. A HP Ultra 2 column, 25 m x 0.20 mm i.d. bonded phase 5% phenylsilicone with 0.33 um film thickness (Hewlett-Packard, Avondale, PA) was used. The initial oven temperature of 90°C was held for 0.5 min, increased to 290°C at 30°C/min and held at 290°C for 20 min. Injector temperature was programmed from 100°C to 290°C at 150°C/min and held at 290°C for 25 min. The detector temperature was 290°C. Helium was used as the carrier gas at a head pressure of 20 psi and 30 ml/min flow rate as a make-up gas. Cholesterol oxides were identified by comparison of their retention times against authentic standards, and their individual response factors were determined relative to 6-ketocholesterol. Peak areas were integrated using a Shimadzu EZChrom chromatography data system (Shimadzu Scientific Instruments Inc., Columbia MD).

To confirm the identity of COP's isolated from oil samples an HP 5890 II Series GC equipped with an on column injector, a 7673 Autosampler and a 5971A mass selective detector was used. Cholesterol oxides were separated on a DB5-MS capillary column (J&W Scientific, Inc., Rancho Cordova, C.A. 95630) 30 m x 0.25 mm i.d. x 0.25um film thickness. The initial column temperature of 80°C was held for 0.5 min, increased to 280° C at 40°C/min and held at 280°C for 18 min. Injector temperature was programmed from 90°C to 280°C at 150°C/min and held at 280°C for 25 min. GC-MS interface temperature was maintained at 280°C and 0.5 ul of sample was injected onto a DB-5 gas chromatography column. Helium was used as the carrier gas at a flow rate

of 1 ml/ min. The analyses were performed with the detector in the scan mode and an electron impact ionization potential at 70 eV.

Statistical Analysis

One-way ANOVA was used to analyze differences in fatty acids of different oil within a storage period. Data for cholesterol oxides and tocopherols were analyzed using three-way analyses of variance with sources of variation of oils (o=4), tocopherol (t=2), and days of storage (s=3). Means of effects (oil, tocopherol and storage) were separated by the Student-Newman-Keuls (SNK) test (Steel and Torrie, 1980). Data related to cholesterol oxides of heated oil were analyzed using two way analyses of variance within heating. Means of interaction were compared using the PDIFF function of the SAS program (SAS Institute, 1985).

2. 3. Results and Discussion

Fatty acid composition of four oils are summarized in Table 2-1. PUFA contents of each oil as a percentage of total fatty acids, were 37.9%, 79.6%, 66.3%, and 10.3% for the fish, flax, sunflower and palm oil, respectively. Fish oil contained mainly long chain polyunsaturated fatty acids (LCPUFA), C20:5n3 and C22:6n3, whereas flax oil and sunflower oil contained mainly C18:3n3 and C18:2n6, respectively. Palm oil contained Palmitic acid (C16:0), and total saturate levels were higher in the palm oil and fish oil. Palm oil was the most saturated oil among those studied. The prominent monoenoic acids

were a mixture of geometric isomers of C16:1 and C18:1 in fish oil whereas the major monoenoic acid was C18:1 in the other three vegetable oils.

Total tocopherol content and major tocopherol isomers were by source of oil (Table 2-2). Flax oil contained a high level of natural tocopherols, ca 890 ppm, especially γ and δ-tocopherol, while fish oil contained only ca 19 ppm. The natural tocopherol contents in order of magnitude were flax oil > sunflower oil > palm oil > fish oil. Sunflower oil and palm oil mainly contain α-tocopherol, 211 and 102 ppm, respectively. The level of tocopherol decreased gradually ($P<0.05$) with storage time (Table 2-3), and after heating (Table 2-4). No detectable tocopherols were detected in the fish oil after 35 days. However, the vegetable oils contained high levels of tocopherols, particularly flax oil, after 35 day storage. The extent of the decline was positively correlated to the initial concentration of tocopherols in the oils, with the greatest losses occurring in oils with the highest initial content with both storage and heating treatments.

Changes in total cholesterol oxides are shown in Table 2-5. Cholesterol oxides were not detected initially in the oils except in fish oil (6-11 ppm). The contents of cholesterol oxides in all oils increased ($P<0.05$) with the time in storage. The fish oil had the largest amount of cholesterol oxides, up to over 30-fold when compared to palm oil at 35 days, followed by sunflower oil. Sunflower oil produced more cholesterol oxides than flax oil during storage even though flax oil has a much higher concentration of oxygen-sensitive polyunsaturated fatty acids, such as 18:3n3. For palm oil, very low levels of cholesterol oxides were formed under this experimental condition. It may be that LCPUFA (C20:5n3 and C22:6n3) greatly influences cholesterol oxidation. The difference

in susceptibility of cholesterol oxidation between fish oil and other vegetable oils may be attributed to different degrees of unsaturation of triacylglycerols. This is supported by the fact that a small amount of cholesterol oxides accumulated in palm oil, which is rich in saturated fatty acids. Ohshima et al (1993) reported that cholesterol oxidation in fish products proceeds in conjunction with oxidative decomposition of coexisting polyunsaturated fatty acids of fish oils. Changes in the concentration of oxidized cholesterol products in the oil occurred during storage but the pattern of these changes differed by the types of cholesterol oxide (Table 2-6). The most prevalent oxide formed as 7-ketocholesterol, which confirmed its use as a marker of cholesterol autoxidation (Maerker, 1987) and epoxides (α and β). This was in accordance with published data (Fontana et al., 1993). As reported by Maerker (1987) the ratios of 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol were about 1:2:6. No detectable level of 25-hydroxycholesterol and cholestanetriol were found in the oil samples during storage.

Tocopherols delay cholesterol oxidation and protect lipid from autoxidation (Rankin and Pike, 1993; Sagers, 1991). The γ- and δ-tocopherols were more effective than α-tocopherol in preventing cholesterol oxidation (Rankin and Pike, 1993). Adding α-tocopherol to oils significantly ($P<0.05$) reduced concentrations of cholesterol oxides in fish oil but had no significant effect on vegetable oils. This might be related to the high levels of natural tocopherol content in vegetable oils (Table 2-2) and suggests that adding tocopherol to vegetable oil may not be necessary to prevent lipid autoxidation. Tocopherols used at relatively low concentrations (100 to 300 ppm of fat weight) are effective antioxidants in a wide variety of food products, and their effectiveness is known

to diminish when used at high levels (>500 ppm of fat weight) (Dougherty, 1988). Jung and Min (1990) demonstrated that optimum concentrations of α-, γ-, and δ-tocopherol increase oxidative stability in soybean oil at 100, 250 and 500 ppm, respectively. Sagers (1991) demonstrated that although α-tocopherol inhibited cholesterol autoxidation in an aqueous model system at 0.02% and 0.2%, but at 2.0% exhibited pro-oxidant properties.

The estimated amounts of cholesterol oxides in unheated and heated oils are summarized in Table 2-7. Unheated oils had no detectable oxides except in fish oil, but after heating, cholesterol oxides were increased up to 22-152 ppm. 7-Ketocholesterol and β-epoxide are predominant in heated oil and heating generates more cholesterol oxides. Oils with tocopherol addition contain significantly ($P<0.05$) lower cholesterol oxides in fish and sunflower oils, but no significant differences were observed in flax and palm oil (Table 2-7). Heated fish oil showed the presence of cholestanetriol (Table 2-7), the most toxic derivative of cholesterol (Blankeship et al., 1991). The heating of cholesterol during cooking favours autoxidation, due to the presence of oxygen, high temperatures, the length of heating time (Smith, 1981) and lipid medium (Bascoul et al., 1983), all of which results in the formation of peroxides. From these studies, there is no doubt that temperature is a strong factor in lipid autoxidation. The present study suggests that both cooking temperature and storage environment are equally important in producing cholesterol oxides in food oils.

In summary, fatty acid composition and tocopherol content in food oils influenced the stability of cholesterol. The level of oxides in oils increased during storage. Fish oil was easily oxidized. The present results indicate that cholesterol oxidation is accelerated

by highly long chain polyunsaturated fatty acids, whereas tocopherols effectively delay cholesterol oxidation. Vegetable oils intrinsically contain tocopherols. Therefore, the beneficial effect of adding extra tocopherols to these oils seems marginal. Products from source that are low or deficient in natural antioxidants like fish oil can be improved by tocopherol supplementation. Heating is a predominant factor in lipid oxidation. Cholesterol oxides formation in commercially processed and stored foods can be prevented by simple addition of vitamin E or a natural form of tocopherols which would benefit the food industry and human health. This implies that antioxidant supplementation in animal diets may decrease cholesterol oxide formation. Further studies on the effectiveness of natural antioxidants are warranted.

Table 2-1. Major Fatty Acid Composition of Food Oils

Fatty acid (%)	Oils								
	FO	FO+T	LO	LO+T	SO	SO+T	PO	PO+T	SEM
C14:0	9.0	9.2	0.0	0.0	0.15	0.13	1.1	1.1	0.12
C16:0	19.0	19.0	5.7	5.5	6.9	6.8	43.2	43.2	0.2
C18:0	3.4	3.4	2.7	2.7	5.0	4.9	4.6	4.6	0.03
C16:1	11.7	12.1	0.05	0.02	0.2	0.2	0.2	0.2	0.08
C18:1n-9	9.4	8.9	11.5	11.4	18.6	18.6	39.0	39.0	0.17
C18:2n-6	1.6	1.4	16.1	16.8	65.0	64.8	9.9	9.9	0.23
C18:3n-3	1.4	1.4	63.4	62.8	0.78	0.7	0.4	0.4	0.2
C18:4n-3	3.5	3.6	0.0	0.0	0.0	0.0	0.0	0.0	
C20:5n-3	14.5	14.7	0.0	0.0	0.63	0.64	0.0	0.0	
C22:6n-3	12.8	12.9	0.0	0.0	0.0	0.0	0.0	0.0	
SAFA	33.7	34.1	8.4	8.2	12.0	11.9	49.0	49.0	0.24
MUFA	28.4	27.8	12.2	12.1	21.6	22.1	40.7	40.8	0.25
Total n-6	2.9	2.8	16.1	16.8	65.0	64.8	9.9	9.9	0.23
Total n-3	35.0	35.3	63.4	62.9	1.4	1.3	0.4	0.4	0.39
PUFA	37.9	38.1	79.5	79.6	66.4	66.2	10.3	10.3	0.31

FO = Fish oil; FO+T = Fish oil + α-Tocopherols; LO = Flax oil; LO+T = Flax oil + α-Tocopherols
PO = Palm oil; PO+T = Palm oil + α-Tocopherols; SO = Sunflower oil; SO+T = Sunflower oil + α-Tocopherols
SAFA = Saturated fatty acids; MUFA = Monounsaturated fatty acids; PUFA = Polyunsaturated fatty acids.
SEM = Standard error of mean.

Table 2-2. Initial Tocopherol Contents in Different Food Oils

Treatments	Tocopherol isomers (ppm)			
	α-T	γ-T	δ-T	Total -T
FO	11.8±1.4*	5.0±0.5	2.1±0.15	18.9±1.8
FO+T	333.9±20	7.3±2.2	3.4±2.1	344.6±20
LO	91.1±5.6	631.6±17	170.9±6.9	893.6±26
LO+T	415.5±10	626.3±21	169.3±4.5	1211.1±25
SO	211.5±5.5	25.2±1.2	0.0	236.7±5.3
SO+T	522.3±6.3	31.3±8.7	0.0	553.6±12
PO	102.3±9.7	7.9±1.7	0.0	110.2±11
PO+T	426.4±6.2	10.3±1.8	0.0	436.7±6.4

*Mean±SD (n=6)
FO = fish oil; FO+T = fish oil + α-tocopherol
LO = flax oil; LO+T = flax oil + α-tocopherol
SO = sunflower oil; SO+T = sunflower oil + α-tocopherol
PO = palm oil; PO+T = palm oil + α-tocopherol
α-T=α-Tocopherol; γ-T = γ-Tocopherol; δ-T = δ-Tocopherol

Table 2-3. Changes of Total Tocopherol Content During Storage in Different Food Oils

Oils	Storage (day) 0	15	35
FO	18.9[a]	1.51[a]	0.0[a]
FO+T	344.6[a]	102.4[b]	0.0[c]
LO	893.6[a]	840.8[a]	681.2[b]
LO+T	1211.1[a]	1135.4[b]	791.7[c]
SO	236.7[a]	215.5[a]	141.0[b]
SO+T	553.6[a]	479.2[b]	374.2[c]
PO	110.2[a]	62.0[a]	62.3[a]
PO+T	436.7[a]	290.8[b]	249.0[b]

FO = fish oil; FO+T = fish oil + α-tocopherol
LO = flax oil; LO+T = flax oil + α-tocopherol
SO = sunflower oil; SO+T = sunflower oil + α-tocopherol
PO = palm oil; PO+T = palm oil + α-tocopherol
a-c Means within same row with no common superscripts differ significantly ($P<0.05$)
Standard error of mean (SEM) was pooled: 19.24

Table 2-4. Changes of Total Tocopherols Contents between Unheated vs Heated Oils

	Oils							
	FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T
	(μg/g oil)							
UnHeated oil	18.0[a]	344.6[a]	896.3[a]	1191.1[a]	110.0[a]	436.7[a]	236.7[a]	553.6[a]
Heated oil	3.7[a]	163.9[b]	665.4[b]	848.8[b]	35.9[b]	219.3[b]	125.5[b]	356.4[b]

PO = Palm oil; PO+T = Palm oil + α-Tocopherols; SO = Sunflower oil;
SO+T = Sunflower oil + α-Tocopherols.
Pooled standard error of mean = 15.18.
a-b Means within same column with no common superscripts differ significantly ($P<0.05$)

Table 2-5. Effects of Storage and α-Tocopherol on Cholesterol Oxidation in Different Food Oils

	Storage (day)			
Oils[1]	0	15	35	overall[2]
FO	11.18^{y}	$62.14^{a,y}$	$133.09^{a,x}$	68.81^{a}
FO+T	8.49^{e}	$41.58^{b,y}$	$73.43^{b,x}$	41.16^{b}
LO	nd	$1.93^{e,x}$	$9.92^{d,x}$	3.95^{de}
LO+T	nd	$1.89^{e,x}$	$8.47^{d,x}$	3.45^{de}
SO	nd	$4.65^{e,y}$	$25.51^{c,x}$	10.05^{c}
SO+T	nd	$3.63^{e,y}$	$21.29^{c,x}$	8.31^{cd}
PO	nd	$2.79^{e,x}$	$4.07^{d,x}$	2.29^{e}
PO+T	nd	$1.28^{e,x}$	$4.38^{d,x}$	1.89^{e}
Overall[3]	2.46^{z}	14.98^{y}	35.02^{x}	

FO = fish oil; FO+T = fish oil + α-tocopherol
LO = flax oil; LO+T = flax oil + α-tocopherol
SO = sunflower oil; SO+T = sunflower oil + α-tocopherol
PO = palm oil; PO+T = palm oil + α-tocopherol
a-d Means within same column with no common superscripts differ significantly ($P<0.05$)
x-y Means within same row with no common superscripts differ significantly ($P<0.05$)
[1]Standard error of mean (SEM) for 8 treatments during storage was pooled : 3.37
[2]Standard error of mean (SEM) for the overall was pooled: 1.95.
[3]Standard error of mean (SEM) for the overall was pooled: 1.19.
nd = not detected

Table 2-6. Effect of Storage and Tocopherols on the Major Cholesterol Oxide Formation in Different Food Oils

	Oils							
COPs(ppm)	FO	FO+T	LO	LO+T	SO	SO+T	PO	PO+T
	Zero day storage							
7α-OH	nd	nd	nd	nd	nd	nd	nd	nd
7β-OH	nd	nd	nd	nd	nd	nd	nd	nd
7-Keto	6.2	6.3	nd	nd	nd	nd	nd	nd
α-Epoxide	1.9	0.9	nd	nd	nd	nd	nd	nd
β-Epoxide	4.4	2.0	nd	nd	nd	nd	nd	nd
	Fifteen day storage							
7α-OH	1.4^{a}	nd	0.3^{d}	0.4^{d}	1.1^{b}	0.7^{c}	0.4^{d}	0.2^{d}
7β-OH	nd	nd	0.1	nd	0.2	nd	0.4	0.3
7-Keto	24.5^{a}	11.6^{b}	0.6^{c}	0.9^{c}	1.8^{c}	1.4	nd	nd
α-Epoxide	23.7^{a}	15.9^{b}	0.5^{c}	1.1^{c}	1.2^{c}	0.9^{c}	nd	nd
β-Epoxide	21.3^{a}	14.0^{b}	0.3^{c}	0.3^{c}	1.0^{c}	0.7^{c}	nd	nd
	Thirty five day storage							
7α-OH	3.3	1.5	1.4	1.0	2.5	1.8	0.5	0.2
7β-OH	2.2	0.7	0.5	0.3	2.0	1.8	0.6	0.2
7-Keto	64.2^{a}	31.9^{b}	3.2^{d}	3.8^{d}	10.8^{c}	10.3^{c}	1.1^{e}	0.8^{e}
α-Epoxide	30.1^{a}	20.8^{b}	1.8^{d}	2.1^{cd}	3.6^{c}	3.4^{c}	1.3^{d}	0.9^{d}
β-Epoxide	34.3^{a}	21.3^{b}	1.8^{d}	1.6^{d}	5.3^{c}	4.3^{c}	0.9^{d}	1.0^{d}

FO = fish oil; FO+T = fish oil + α-tocopherol
LO = flax oil; LO+T = flax oil + α-tocopherol
SO = sunflower oil; SO+T = sunflower oil + α-tocopherol
PO = palm oil; PO+T = palm oil + α-tocopherol
7α-OH: 7α-Hydroxycholesterol; 7β-OH: 7β-Hydroxycholesterol;
7-Keto:7-Ketocholesterol
a-e Means within same row with no common superscripts differ significantly ($P<0.05$)
nd = not detected

Table 2-7. Effects of Heating and Tocopherols on Cholesterol Oxidation in Different Food Oils

Oils	Total COPs	Major COPs* (ug/g) 7α-OH	7β-OH	α-Epoxide	β-epoxide	triol	7-keto
			Unheated oils				
FO	11.6	nd	nd	1.2	4.3	nd	6.0
FO+T	9.1	nd	nd	0.8	2.1	nd	6.1
LO	nd	nd	nd	nd	nd	nd	nd
LO+T	nd	nd	nd	nd	nd	nd	nd
SO	nd	nd	nd	nd	nd	nd	nd
SO+T	nd	nd	nd	nd	nd	nd	nd
PO	nd	nd	nd	nd	nd	nd	nd
PO+T	nd	nd	nd	nd	nd	nd	nd
			Heated oils				
FO	152.2^{a}	16.8^{a}	15.4^{a}	31.2^{a}	56.5^{a}	4.4	32.4^{a}
FO+T	75.2^{b}	6.1^{b}	6.1^{b}	15.7^{b}	32.0^{b}	2.7	15.3^{c}
LO	28.5^{de}	2.3^{c}	1.5^{d}	6.8^{cd}	11.4^{cd}	nd	6.5^{de}
LO+T	22.2^{e}	2.0^{c}	3.7^{c}	5.5^{cd}	8.8^{de}	nd	2.3^{e}
SO	56.5^{c}	3.6^{c}	3.0^{cd}	7.7^{c}	15.9^{c}	nd	26.4^{b}
SO+T	40.0^{d}	3.4^{c}	2.8^{cd}	7.7^{c}	12.6^{cd}	nd	13.5^{c}
PO	31.3^{de}	2.1^{c}	2.0^{cd}	4.9^{cd}	6.2^{ef}	nd	16.0^{c}
PO+T	22.0^{f}	2.1^{c}	1.7^{d}	3.3^{c}	3.6^{f}	nd	11.4^{cd}
SEM	4.39	0.66	0.62	1.21	1.65		1.79

* COPs = Cholesterol oxidation products
FO = Fish oil; FO+T = Fish oil + α-Tocopherol; LO = Flax oil; LO+T = Flax oil + α-Tocopherol;
PO= Palm oil; PO+T = Palm oil + α-Tocopherol; SO = Sunflower oil; SO+T = Sunflower oil + α-Tocopherol.
7α-OH: 7α-Hydroxycholesterol; 7β-OH: 7β-Hydroxycholesterol; 7-Keto: 7-Ketocholesterol;
25-OH: 25 hydroxycholesterol;
SEM = Standard error of mean
a-f Means within same column with no common superscripts differ significantly ($P<0.05$)
nd = not detected

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Chapter 3. COPs Content of Egg yolk Powder: Methodology and Influencing Factors

3. 1. Isolating Cholesterol Oxides by Silicic Acid Column and Analysis Using Selected Ion Monitoring (SIM)

3. 1. 1. Introduction

Concern regarding cholesterol autoxidation in food is growing because of the link between cholesterol oxides and atherosclerotic injury (Imai et al., 1976, 1980; Peng et al., 1978, 1979; Taylor et al., 1979; Blankenship et al., 1991). The separation and quantification of cholesterol oxides in foods have been difficult due to their trace concentrations and chemical similarity to cholesterol. A number of methods have been developed for the quantitative determination of cholesterol oxides in various food products exposed to heat and air during manufacturing and/or long term storage, but no standard method exists for accuracy, precision, and selectivity analysis of COPs in foods (Nourooz-Zadeh, 1990). All methods for analysis of cholesterol oxides from tissues and foods require concentration of the more polar cholesterol oxides by removal of extraneous lipids and the bulk of cholesterol. The separation of cholesterol oxides has been previously achieved by saponification of the extracted lipids with hot alkali (Naber and Biggert, 1985; Finocchiaro and Richardson, 1984). This step frees the bulk of the lipids from the sterol residue and also converts esterified cholesterol to the free sterol. However, hot

alkaline treatment of cholesterol and its autoxidation products is a significant source of error in the quantification of COPs (Finocciaro and Richardson, 1983; Nourooz-Zadeh, 1990). Smith (1981) reported the artifactual formation of cholesterol oxidation products during saponification. Tsai et al. (1980) reported a loss of 75% of the α-epoxide after saponification.

Researchers have developed a method for enriching COPs using silicic acid column to remove the neutral lipids and cholesterol, followed by TLC to clean up samples, and finally capillary gas chromatography analysis of COPs (Park and Addis, 1985; Zubillaga and Maerker, 1991). However, the separation of COPs by TLC is lengthy and troublesome, and also allows exposure of the samples to potential oxidation as a result of its large surface area (Smith and Hill, 1972; Smith et al., 1967; Park and Addis, 1985; Chen et al., 1994). These methods were not suitable for our purposes for artifact, and lack of specificity and sensitivity.

The objective of this study was to describe a method for the quantitative determination of some cholesterol oxidation products that would be found in dried egg powder products. It employs an isolation and prefractionation step using silicic acid column chromatography to remove neutral lipids and cholesterol, and finally selected ion monitoring (SIM) analysis of cholesterol oxides as trimethylsilyl ether (TMS ether) derivatives.

3. 1. 2. Materials and Methods

Reagents

Cholesterol and cholesterol oxide standards (> 99% purity), listed in Table 3-1, were purchased from Sigma Chemical Co. (St Louis, MO) and Steraloids Inc. (Bayonne, New Hampshire). Celite 545 and Calcium phosphate ($CaHPO_4 \bullet 2H_2O$) were purchased from Fisher Scientific Co. (Malvern PA) and silicic acid (100 mash) from Aldrich Chemical Co. (Milwaukee, WI). All solvents used were glass distilled.

Egg yolk powder samples

Egg yolk powders were prepared by using a Mini Spray Dryer (Buchi Laboratoriums-TE Chnik AG. CH-9230, Flawil, Switzerland) with an inlet temperature of 128°C and an outlet temperature of 70°C from eggs of hens which had been fed a complete basal diet containing a 3.5% of fish oil (FO), flax oil (LO), palm oil (PO), or sunflower oil (SO) with and without 0.07 % antioxidant (Mixed tocopherols (T). The mixed tocopherol (Henkel Corp., Kankekee, IL 60901-0191) contained 17.3%, 36.3%, 7.3% and 60.9% of δ, γ, α and total tocopherols, respectively. Equal aliquots were taken in triplicate from each of eight batches of egg powder and stored in polythene containers at room temperature, and open to air. The samples were shaken daily to ensure uniformity of light (normal daylight cycle) and air access. Small aliquots were taken from each of the triplicate samples of the eight batches of powder and analyzed after 0, 1, 2, 3 and 4 months of storage.

Extraction of lipids

The method of Folch et al. (1957) was employed for extraction of lipids. Briefly, to 0.5 g samples, 10-50 ug 6-ketocholestanol were added as an internal standard. The sample was homogenized in 20 ml of chloroform ($CHCl_3$)/methanol (CH_3OH) (2:1, v/v) vortexed for 2 min, and filtered through glass wool. Another 20 ml $CHCl_3$/ CH_3OH (2:1, v/v) were used to rinse the tubes and to reextract the residue. Combined filtrates were washed with 8 ml distilled water under nitrogen, kept in a cold room over night, and the organic layer was then collected. The dried total lipid extracts were obtained after evaporating the solvent under nitrogen.

Sample preparation

A column chromatography method for isolation of cholesterol oxides (Park and Addis, 1985; Zubillaga et al., 1991) was used with some modifications. A homogenous mixture containing silicic acid (100 mesh), Celite 545 (Fisher Scientific) and $CaHPO_4 \bullet 2H_2O$ (10:9:1 wt/wt/wt) was made into a slurry with chloroform, and packed into a glass column (12 mm i.d. x 30 cm) to a height of 15 cm. The column was washed with 10 ml hexane:ethyl acetate (9:1 v/v, solvent-1) before samples were applied. Lipid extracts, redissolved in 5 ml of 9:1 hexane / ethyl acetate (v/v), were applied onto the column. Neutral lipids and cholesterol were eluted by passing 50 ml solvent-1 and then 40 ml hexane:ethyl acetate (8:2 v/v, solvent-2) through the column. Elution of cholesterol oxides was accomplished with 40 ml of acetone:ethyl acetate:methanol (50:50:5 v/v/v, solvent-3) at approximately 1 ml/min flow rate. Twenty (20) ug of 5α-cholestane was

added to the final elute, then dried under nitrogen and cholesterol oxidation products were converted to TMS derivatives with 200 ul pyridine and 100 ul sylon BFT (Supelco Inc., Bellefonte, PA) at 80°C for 1 h.

GC-MS Analysis

Analysis of cholesterol oxides was performed on an HP 5890 II Series GC equipped with an on-column injector, a 7673 Autosampler and a 5971A Mass Selective Detector (MSD). Separation was achieved using a DB5-MS capillary column (J&W Scientific, Folsom, C.A. 95630) 30 m x 0.25 mm i.d. x 0.25 um film thickness. The initial column temperature of 80°C was held for 0.5 min, increased to 280°C at 40°C/min and held at 280°C for 18 min. Injector temperature was programmed from 90°C to 280°C at 150°C/min and held at 280°C for 25 min. GC-MS interface temperature was maintained at 280°C and 0.5 ul of sample was injected onto a DB-5 gas chromatography column. Helium was used as the carrier gas at a flow rate of 1 ml/ min. The MS was operated in the electron impact mode with an electron impact ionization electron voltage at 70eV.

Identification of individual TMS oxides was made with the MS in the scan mode by comparison of the GC peak retention times and mass fragmentation data against those authentic standards. Quantification was made using a selected ion monitoring (SIM) mode with the following ions measured: m/z 217 for 5α-cholestane, m/s 456.35 for 7α- and 7β-hydroxycholesterol, m/z 197.1 for α- and β-epoxides; m/z 403.4 for cholestanetriol, m/z 445.3 for 6-ketocholestanol, m/z 367.35 for 7-ketocholesterol, m/z 271.2 for 25-

hydroxycholesterol. Run time was 23 mim with a dwell time of 100 msec per ion. Peak areas of the ions monitored were measured using the HP Chemstation software. The amount of oxides formed was quantitated from the ratio of the peaks at m/s corresponding to individual COPs and m/z 445.3 of internal standard 6-ketocholestanol.

Recovery Estimates

A standard mixture of known amounts (15 ug) of each cholesterol oxidation product, internal standard and 200 ug of cholesterol was carried through the whole procedure. The same COPs standards were added in known amounts (7.5 ppm) to a fresh egg powder sample through the whole procedure, and analyzed under identical conditions as the egg yolk powder samples. Recovery calculation was based on 5α-cholestane, added in the last step, by dividing the amount of standards found by the amount calculated from the direct analysis of same standard mixture after derivatization. A response factor for each oxide was obtained by dividing peak area ratio by weight ratio of oxide to 6-ketocholestanol in the prepared standard mixture.

Statistical analysis

Data were analyzed using three-way analyses of variance with sources of variation of oils (o=4), tocopherol (t=2), and days of storage (s=5), Means of effects (oil, tocopherol and storage) were separated by the Student-Newman-Keuls (SNK) test (Steel and Torrie, 1980). Means of interaction were compared using the PDIFF function of the SAS program (SAS Institute, 1985).

3. 1. 3. Results & Discussion

The method used to isolate and measure cholesterol oxidation products is outlined in Fig. 3-1. Before quantification, the cholesterol oxidation products had to be freed from the bulk lipid of egg powder. About 60% of the egg yolk solids are lipids, approximately 4% of which are cholesterol and 27% of which are phospholipids (Tsai and Hudson, 1984). To prevent oxidation of cholesterol during analysis, a minimum number of sample clean-up steps is desirable. Saponification has been used to concentrate sterol fractions and separate it from other lipids (Park and Addis, 1987; Pie et al., 1990; Wahle et al., 1993). However, alkaline treatment can cause significant loss in the quantification of 7-ketocholesterol even with mild saponification (Van de Bovenkamp, 1988) and α-epoxide (Tsai et al., 1980). TLC has also been used to fractionate lipid classes and isolate sterols (Zubillage and Maerker, 1991; Park and Addis, 1985). In addition to being time consuming, we experienced a significant increase in all measured cholesterol oxides in the samples initially separated by TLC compared to those without TLC preparation. To account for possible losses of cholesterol oxides during sample preparation, internal standard was added at the initial step of sample preparation. Althougth Park and Addis (1985) used only acetone to elute cholesterol oxides, acetone was not effective for triol recovery in the present study. Fifty ml of solvent III (acetone:ethyl acetate:methanol 50:50:5, v/v/v) was needed to elute all sterol oxides. It was found that using the silicic acid column and three different solvent systems was an adequate sample clean-up when combined with an efficient capillary column for separation.

Figure 3-2 and Figure 3-3 represent the total ion chromatography profile of the

TMS derivatives from a standard mixture and an egg powder sample, respectively, using the SIM mode. All of the major cholesterol oxides were fully resolved except for 25-hydroxycholesterol and 6-ketocholestanol which co-eluted. Although the retention times were the same, accurate quantitation was achieved by measuring 25-hydroxycholesterol at m/z 271.2, (this mass was absent in the mass spectra of 6-ketocholestanol) and 6-ketocholestanol at m/z 445.3 using the selected ion monitoring. 25-Hydroxycholesterol was not detected in egg yolk powders during storage. The advantages of SIM mode are greater sensitivity, and more accurate quantitation of overlapping peaks in a complex sample by monitoring ions characteristic of each peak.

To test the procedure, known amounts of cholesterol oxide standard mixture (purchased standards) and 200 ug of cholesterol were taken through the full procedure. Using 5α-cholestane as an internal standard, added after the column preparation step, the overall recoveries were between 86.13% and 99.66% (Table 3-2). The same oxides were added in known amounts to fresh egg powder. Recoveries (Table 3-2) varied between 81.48% and 109.67%. Cholestanetriol was the only one demonstrating a low recovery range at 81.4-86.13%. This suggests that clean-up of the sample through the silicic acid column was very effective in separating and concentrating cholesterol oxide from other lipids.

The cholesterol oxide contents of dried egg yolk powder, stored at room temperature for up to four months are presented in Figures 3-4 and Figure 3-5. All egg yolk powder samples contained at least five cholesterol oxidation products of interest at the detection limit of 0.15 ppm. Cholesterol oxides significantly ($P<0.05$) increased with

time of storage in all treatments. 7-ketocholesterol and β-epoxide were the most abundant individual cholesterol oxidation products in dried egg yolk powder of eight treatment diets throughout storage. Fontana et al. (1993) reported that β-epoxide was the predominant derivative with aged egg powder and 7-ketocholesterol was predominant in heated egg powder. The stability of α-epoxide is also interesting because it can be converted to cholestanetriol by hydrolysis. The epoxides were stable in this experimental condition and no detectable cholestanetriol was found. The epoxides are reported as non-atherogenic but can be hydrolyzed by stomach acid to produce the very atherogenic cholestanetriol (Missler, 1985). However, there is some evidence indicating that the epoxides may be carcinogenic (Black and Douglas, 1972). 7-Ketocholesterol, 7α-hydroxycholesterol and 7β-hydroxycholesterol are moderately atherogenic (Peng et al., 1979). Therefore, their occurrence may be a cause for concern.

In summary, an effective, sensitive and convenient method was developed for concentrating and determining the cholesterol oxidation products in egg yolk powder. Selected ion mass spectrometric analysis is very useful for quantitation of cholesterol oxides in complex mixtures where coeluting peaks are present. The present method eliminates lengthy steps of saponification and TLC, thus minimizing artifact formation in dried egg yolk samples during analysis and allowing greater accuracy and sensitivity.

3. 2. Effect of Dietary Oils and Tocopherol Supplementation on Lipid and Cholesterol Oxidation in Egg Yolk Powder during Storage and Heating

3. 2. 1 Introduction

Chicken eggs, due to their relatively high cholesterol content, have been singled out by the diet-heart advocates as food to be avoided (Connor and Connor, 1983) even though the egg contains the best and least-expensive high quality protein and a balanced distribution of minerals and vitamins for maintaining human health (Shrimpton, 1987; Moreng and Avens, 1985). The per capita consumption of egg has been declining in western countries. The major cause is consumers' increasing health consciousness of diet and the controversial relationship between dietary cholesterol and coronary heart disease (CHD) (Connor and Connor, 1983; Steinberg, 1989; Health and Welfare Canada, 1990). Despite tremendous research efforts, the role of dietary cholesterol and plasma and lipoprotein cholesterol levels in coronary heard disease and atherogenesis remains uncertain (Steinberg, 1989). Recently, the interest in the link between cholesterol oxidation products and atherosclerosis has intensified (Hubbard et al., 1989; Peng and Morin, 1992). This notion was supported by the finding (Imai et al., 1976) that cholesterol oxides from USP (United States Pharmacopoeia) grade cholesterol produced angiotoxicity and arteriosclerosis in rabbits, while purified cholesterol did not. Cholesterol is an unstable compound that undergoes autoxidation in the presence of molecular oxygen and

light, through a free-radical reaction. More than 60 autoxidation products have been reported (Smith, 1981). These cholesterol oxidation products (COPs) are formed in food containing cholesterol subjected to common processing conditions such as spray-drying, deep-fat frying, and during prolonged storage. COPs have been identified in a variety of foods including raw, cooked, and dehydrated meats (Sander et al., 1989), dairy products (Cleveland and Harris, 1987), fish products (Ohshima et al., 1993; Li et al., 1994) and egg products (Naber and Biggert, 1985; Addis, 1986; Wahle et al., 1993). Factors such as storage time and exposure to light, air, or cooking temperature can have significant effects on the production of COPs in foods.

Attempts to reduce the cholesterol content of eggs were not successful (Naber, 1983; Hargis, 1988). However, it is known that fatty acid composition, fat soluble vitamins, certain trace minerals such as iodine and selenium, and vitamin B_{12} content can be manipulated (Hill et al., 1961; Jiang et al., 1994; Squires, and Naber, 1992). Moreover, the cholesterol-lowering and ameliorative effects against atherosclerosis of dietary n-3 PUFA are of particular interest to egg producers, consumers, and researchers. It is reported that enrichment of chicken egg (Sim, 1990) with omega-3 fatty acids can be achieved by incorporating flax and canola seeds or their oils in laying hen diets. However, markedly increasing the level of polyunsaturated fatty acids including omega-3 fatty acids in poultry products could increase lipid oxidation. Fritsche and Johnson (1988) identified the following as possible health risks associated with increased consumption of omega-3 fatty acids; 1) increased consumption of lipid oxidation products, 2) increased in vivo production of lipid oxidation products, and 3) depletion of tissue levels of vitamin E.

Cholesterol autoxidation is a free radical mechanism, similar to autoxidation of unsaturated fatty acids (Nawar, 1985). To increase nutritional value and safety, preventing cholesterol oxidation during storage and cooking has become a critical need. Tocopherols as antioxidants have been used successfully to delay cholesterol oxidation in aqueous meat model system (Rankin and Pike, 1993). It is also well established that α-tocopherol supplementation of poultry diets improved the stability of meat (Sklan et al., 1983; Sheldon, 1984; Lin et al., 1989; Asghar et al., 1990) and eggs (Jiang et al., 1994). The previous chapter demonstrated that the type of lipids (saturated versus polyunsaturated fatty acids) greatly influenced cholesterol oxidation in food oils, and delayed COPs formation by tocopherol supplementation in vitro. There is, however, little information about stabilized cholesterol oxidation in fatty acid-modified eggs. The objective of this study was to investigate the effects of varying fatty acid composition and tocopherol content of dried egg yolk powders, through dietary supplementation of tocopherols to laying hen's diets, on the subsequent susceptibility of egg yolk powders to cholesterol oxide formation and lipid oxidation during storage at room temperature and with cooking.

3. 2. 2 Materials and Methods

Reagents

Cholesterol and cholesterol oxide standards (> 99% purity), including 7α-hydroxycholesterol, 7β-hydroxycholesterol, α-epoxide, β-epoxide, cholestanetriol, 7-ketocholesterol and 6-ketocholestanol, were purchased from Sigma Chemical Co. (St Louis, MO) and Steraloids Inc. (Bayonne, New Hampshire). 5α-cholestane, Butylated

hydroxyltoulene (BHT) and tocopherol isomer standards were purchased from Sigma Chemical Co. (St Louis, MO). All standards were used as purchased. Celite 545 and Calcium phosphate ($CaHPO_4 \bullet 2H_2O$) were purchased from Fisher Scientific Co. (Malvern PA) and silicic acid (100 mash) from Aldrich Chemical Co. (Milwaukee, WI). Acetonitrile and methanol were HPLC grade from BDH Inc., (Toronto, Ontario) and all other solvents used were glass distilled.

Experimental Design

Single Comb White Leghorn pullets, 22 wk of age, were housed in two double-deck cage batteries with two birds in each cage (.31 x .40 m). Each battery had 120 cages divided into 8 units with 15 cages per unit, thus generating a total of 16 experimental units. The birds were allotted to eight dietary treatments. The experimental diets (Table 3-3) contained 3.5% of fish oil (FO), flax oil (LO), palm oil (PO), or sunflower oil (SO), with and without 0.07 % antioxidant diet (Mixed tocopherols (T). The mixed tocopherol (Henkel Corp., Kankekee, IL 60901-0191) contained 17.3%, 36.3%, 7.3% and 60.9% of δ, γ, α and total tocopherols, respectively. The fatty acid composition and tocopherols of the yolks were monitored, and the changes in fatty acid composition of yolks were found to reach a plateau two weeks after feeding the test diets. Ten dozen eggs laid during the sixth week were collected from each treatment. Fifty eggs from each treatment were randomly selected and yolks were separated and completely homogenized. Egg yolks were dried by using Mini Spray Dryer (Buchi Laboratoriums-TE Chnik AG. CH-9230, Flawil, Switzerland) with an inlet temperature of 128°C and an outlet temperature of

70°C. Three equal aliquots were taken from each of eight batches of egg powder and stored in polythene containers at room temperature and open to air. The samples were shaken daily to ensure uniformity of light (normal daylight cycle) and air access. Small aliquots were taken from each of the triplicate samples of eight batches of powder and analyzed after 0, 1, 2, 3 and 4 months of storage. For heating treatment, equal aliquots were taken in triplicate from each of eight batches of egg powder, heated in an air convection oven at 110°C for 22 hrs and subjected to the following analyses.

Fatty Acid Composition Analyses

Total lipids were extracted from egg yolk powder according to Folch et al. (1957). Aliquots of the lipid extracts were dried under nitrogen and were converted to fatty acid methyl esters using a mixture of boron trifluoride, hexane and methanol (35:20:45, v/v/v) (Metcalfe and Schmitz, 1961). Fatty acid methyl esters were separated and quantified by gas chromatography as described in Chapter 2.

Cholesterol Assay

Samples of about 0.1 g of egg yolk powder were saponified according to Fenton and Sim (1991). The unsaponifiable fraction was extracted using 10 ml of hexane, and the hexane extracts were assayed for cholesterol by gas chromatography (GC). Two mg of 5α-cholestane (Sigma Chemical Co., St. Louis, MO 63178) were added to each yolk powder sample as internal standard (IS) before saponification. A DB-5 (J & W Scientific, Folsom CA) 15m x 0.53 mm i.d. x 1.5 μm film thickness fused silica capillary column

was used on a Varian 3400 gas chromatography (Varian Associates, Inc., Sunnyvale, CA 94089) equipped with an automatic sampling system. The operation conditions of GC were as follows: oven temperature was held at 90°C for 0.04 min., then programmed at 25°C/min to 290°C and held at 290°C for 10 min. Injection temperature was programmed from 100 to 290°C at 150°C/min and held at 290°C for 16 min. Liquid CO_2 was used to cool the injector. Injection volume was 0.5 μm at a fast injection rate. Helium carrier-gas head pressure was set at 20 p.s.i. (ca 1.5 ml/min) and a flow rate of 30 ml/min as a make-up gas. Hydrogen and air gas flow to the detector were 30 and 300 ml/min, respectively. Detector temperature was at 290°C. Peak areas were integrated using a Shimadzu EZChrom chromatography data system (Shimadzu Scientific Instruments Inc., Columbia MD).

Tocopherol Assay

Tocopherols were extracted according to Zaspel et al. (1983), with minor modifications. The yolk powder samples (ca. 100-200 mg) were weighed in duplicate, and 200 ul of the internal standard (rac-5,7-dimethyltocol, 10ug/ml in methanol) and 5ul of BHA (1 mg/ml in ethanol) were added, and homogenized in 8 ml of acetone for 2 min with glass beads by vortex. The homogenate was centrifuged at 2000 rpm in a Beckman centrifuge (Beckman Instruments, Mississauga, Ontario, Canada) for 10 min. and the supernatant was transferred to a test tube. The pellet was reextracted with 4 ml of acetone. Both fractions were pooled and dried under N_2 at 30-40°C and redissolved with 2 ml of methanol. A Varian 5000 liquid chromatography system with a Shimadzu Sil-9A

model autosampler was used to determine α, γ and δ-tocopherol concentrations in the yolk powder samples. Separation was achieved on a 3μm Supelcosil RPLC-18 column, 15 cm x 4.6 mm i.d. (Supelco Canada Ltd., Oakville, Ont.) with a guard column (5 cm, 20-40 μm LC-18 packing) and a mobile phase of methanol:acetonitrile (50:50 v/v) at a 1.5 ml/min flow rate. A 25 ul volume was injected to the HPLC column. Tocopherol isomers were detected with a Shimadzu RF-535 fluorescence detector at an excitation wavelength of 295 nm and an emission wavelength of 330 nm. A Shimadzu EZChrom chromatography data system (Shimadzu Scientific Instruments Inc., Columbia MD) was used to integrate peak areas.

Iatroscan TLC-FID of Neutral and Polar Lipids

An Iatroscan TH-10 TLC Analyzer MK II (#93804 Iatron Laboratories, Tokyo, Japan) equipped with a flame ionization detector was used to quantify the neutral and polar lipid classes in the egg yolk powder. Prior to usage, SIII chromarods were burned twice to ensure activation and proper cleaning of the rods. A 5 ul syringe was used to apply 2 ul of sample onto each of the chromarods. Prior to development, chromarods were placed in a constant humidity chamber (30% RH) for 10 min over a saturated solution of sodium carbonate (Ackman et al., 1990) and then transferred immediately into a developing tank containing benzene (65 ml), chloroform (20 ml), and formic acid (0.5 ml) for 1 h. Chromarods were removed from the tank, oven-dried for 2 min at 110°C, and then scanned. The FID separating conditions were: air flow rate 2000 ml/min, hydrogen head pressure gauge set at 0.75 kg/cm^2. The area under the curves for each of

the individual peaks was integrated using a Shimadzu EZChrom chromatography data system (Shimadzu Scientific Instruments Inc., Columbia MD) and then expressed as a percentage of the total area. Identifications were done with commercial standards (Sigma Chem. Co., St. Louis, MO), and response factors were calculated using known concentration and peak areas for standards.

Cholesterol Oxidation Products Analyses

Cholesterol oxides were isolated by column chromatography method and determined qualitatively and quantitatively by selected ion monitoring (SIM) as previously described in Chapter 3. 1.

Statistical Analysis

One-way ANOVA was used to analyze differences in fatty acids among diets within each storage treatment. Data for cholesterol oxides and tocopherols were analyzed using three-way analyses of variance with sources of variation of oils (o=4), tocopherol (t=2), and days of storage (s=5). Means of effects (oil, tocopherol and storage) were separated by the Student-Newman-Keuls (SNK) test (Steel and Torrie, 1980). Data related to cholesterol oxides of cooked powder were analyzed using two way analyses of variance within heating. Means of interaction were compared using the PDIFF function of the SAS program (SAS Institute, 1985).

3. 2. 3. Results and Discussion

Levels of different fatty acids in oil based diets were proportional to levels of dietary incorporation of each oil (Table 3-4). Palmitic acid (C16:0), stearic acid (C18:0) and total saturates levels were higher in the palm oil and fish oil diets than in the flax oil and sunflower oil diets. Fish oil, flax oil and sunflower oil diets contained similar levels of oleic acid (C18:1). The sunflower oil diet contained higher levels of linoleic acid (LA, C18:2n6) and total omega-6 fatty acids, and the flax oil diet contained higher levels of α-linolenic acid (LNA, C18:3n3) and total omega-3 fatty acids. The fish oil diet contained higher levels of long chain polyunsaturated fatty acids such as C20:5n3 and C22:6n3. Cruickshank (1934) reported that yolk fatty acids can be easily altered by dietary manipulation. The fatty acid composition of egg yolk powder was significantly modified by laying hen test diets (Table 3-5). Feeding flax oil and palm oil enriched the egg yolk powders with LNA and oleic acid (18:1n9), respectively. Incorporation of sunflower oil resulted in a significant increase in 18:2n6 and arachidonic acid (20:4n6). Conversely, significantly higher levels of long chain polyunsaturated fatty acids (LCPUFA) such as EPA and DHA were found in egg yolk powders from the fish oil diet than from other diets. Fatty acid profiles of egg yolk powder from eight treatments after stored four months of storage are presented in Table 3-6. Total omega-3, omega-6 and PUFA were decreased in all the treatments after four month storage (compare Table 3-5 and Table 3-6).

Total tocopherol contents of egg yolk powders stored at room temperature for four months and after heating are presented in Table 3-7 and Table 3-8. Egg yolk powder

tocopherol levels of the supplemented animals were 3-4 times greater than for non-supplemented hens ($P<0.05$). Dietary oils had significant ($P<0.05$) influence on the deposition of tocopherols in the egg yolk powder. The prolonged storage of the powders led to a significantly ($P<0.05$) gradual decline in total tocopherol content with time over the four month period and after heating. The extent of the decline was positively correlated to the initial concentration of tocopherols in the powder, with the greatest losses occurring in egg yolk powders with the highest initial content. These results are in agreement with previously reported research (Wahle et al., 1993).

Results for mean total neutral lipid (including triglycerides (TG), cholesterol and diglycerides (DG)) and polar lipid content of egg yolk powder after heating and during a four month storage are shown in Table 3-9 and Table 3-10. No significant differences ($P>0.05$) were found in neutral and polar lipids in heating treatment. After four months of storage, polar lipids significantly ($p<0.05$) increased concomitantly with an decrease in neutral lipids, and the egg yolk powder from birds fed the palm oil diet was significantly ($P<0.05$) lower in polar lipids than the other three dietary oil diets. The egg powder from birds fed supplemented tocopherol showed a significantly ($P<0.05$) lower percentage of polar lipids than the egg powder from birds fed a diet without tocopherol supplementation. These results show that a change in neutral and polar lipid ratio may indicate the quality of food during storage. Iatroscan thin layer chromatography-flame ionization detection (TLC-FID) is a major improvement for rapid quantitative measurement of lipid classes (Ackman et al., 1990; Fraser et al., 1985; Shantha, 1992). It has been reported that changes in neutral and/or polar lipid content could be used as

indicators of lipid oxidation (Kaitaranta and Ke, 1981; Fraser et al., 1985).

Data relating to the cholesterol oxidation products (COPs) content of egg yolk powder, stored at room temperature for up to four months are shown in Table 3-11. Initial levels of cholesterol oxides were only 7-11 ppm and significantly ($P<0.05$) increased with time of storage in all treatments. Previous studies indicate that cholesterol in food (egg, milk, butter) undergoes oxidation when stored for prolonged periods (Nourooz-Zadeh and Appelqvist, 1987; 1988a,b; Tsai and Hudson, 1985; Van de Bovenkamp, 1988) or when exposed to fluorescent light or diffuse daylight (Luby et al., 1986a,b; Sander et al., 1989; Van de Bovenkamp, 1988; Fontana et al., 1993). The COPs content in egg yolk powder in order of magnitude was FO>LO>SO>PO, but no significant differences were observed between flax and sunflower oil diet. The differences in susceptibility of cholesterol oxidation between the fish oil diet and other vegetable oil diets may be attributed to different degrees of unsaturation. Long chain polyunsaturated fatty acids (LCPUFA) such as C20:5n3 and C22:6n3 greatly influence cholesterol oxidation. Li et al (1994) demonstrated that sardine oil triacylglycerols with cholesterol contained 920 ppm of oxides while fully hydrogenated triacylglycerols with cholesterol samples had no oxide content. Cholesterol oxides were significantly ($P<0.05$) lower in tocopherol-supplemented treatments during storage. Cholesterol oxide contents in yolk powder with tocopherol were 30% less than in egg yolk powder without tocopherol from fish oil and flax oil diets. Similarly, in the tocopherol added (FO+T, LO+T, SO+T and PO+T) diets, PO+T treatment had the lowest level of oxides (130 ppm) even though no significant differences were observed among FO+T, LO+T and SO+T treatments. It seems that tocopherols

together with saturated fatty acids offered more protection against cholesterol autoxidation.

Analyses of dried egg yolk powders were compared before and after heating at 110°C for 22 hrs (Table 3-12). Unheated egg yolk powder contained only 6-11 ppm in all eight treatments but after heating, COPs dramatically increased to 150-290 ppm. The egg yolk powder from fish oil without tocopherol diets contained the highest oxides (296 ppm), followed by flax oil diets. Cholesterol oxides were significantly ($P<0.05$) decreased in egg yolk powder from oil with tocopherol supplementation diets, except for sunflower oil diets. The predominant oxides were 7-ketocholesterol and β-epoxide. Note that only the fish oil and flax oil treatment samples showed the presence of 25-OH, the most toxic derivative of cholesterol (Imai et al., 1976; Blankeship et al., 1991). Heating causes both an increase in reaction rate and in direct decomposition of hydroperoxide in heated egg powder (Pie et al., 1990; Naber and Biggert, 1985).

Increasing intrinsic tocopherol concentrations in egg yolk powders by supplementing the laying hen's diet, as in the present study, clearly ameliorated the extent of cholesterol oxidation on prolonged storage and heating. Recent epidemiological (Gey, 1986, 1990), experimental (Steinberg and Witztum, 1990) and pathological evidence (Mitchinson et al., 1990) has implicated low antioxidant status and free-radical-induced lipoprotein peroxidation in the aetiology of atherosclerosis. A hypothesis is supported by the occurrence of a variety of oxidized lipids, including COPs and 'ceroid' material in plaques (Mitchinson et al., 1990) and circulating antibodies to oxidised low density lipoproteins in patients with vascular disease (Steinberg and Witztum, 1990). However,

COPs are also of exogenous dietary origin. They are transported in plasma lipoproteins and are cytotoxic in vivo and vitro (Peng et al., 1987a, b; Hubbard et al., 1989). Clearly, preventing the formation of COPs in foods is of major importance. From our study, tocopherols may play a function of antioxidation of cholesterol by simply increasing the intrinsic tocopherol concentration in egg yolk powder.

Effects of dietary oils, tocopherols, storage, and heating on the loss of cholesterol and formation of its oxides are shown in Table 3-13 and Table 3-14. Approximately 50% cholesterol was lost, compared to initial contents of samples in storage and 0.5-1 % in heated samples. Cholesterol oxides can reach 0.6-2.8% of total cholesterol in storage treatments, and 0.7-1.2% of total cholesterol in heated treatments. These results are consistent with those previously reported by Pie et al. (1990). A major portion of cholesterol decomposition may have occurred via a different pathway, e.g., polymerization (Nawar et al., 1991). From this result, cholesterol oxide formation may be accelerated by high temperature. It is also possible that a considerable amount of produced oxides may have undergone further breakdown at high temperature.

In summary, from our results cholesterol oxide levels in egg yolk powder were affected by temperature, daylight and storage. Temperature is the predominant factor of lipid oxidation. Fatty acid composition and tocopherol content in powders influenced the stability of cholesterol. Egg yolk powder from laying hens fed the fish oil was easily oxidized. The present results indicate that cholesterol oxidation is accelerated by the presence of highly long chain polyunsaturated fatty acids. Feeding tocopherol supplements to laying hens is a novel way of increasing its intrinsic concentration in the eggs and in

subsequent products. The possible beneficial effects of supplementing the diets of laying hens with tocopherols warrants further investigation. Iatroscan TLC-FID lipid analysis showed that percent of polar lipids increased with a decrease in neutral lipids with long storage at room temperature.

Figure 3-1. Isolation and Measurement of Cholesterol Oxidation Products in Egg Yolk Powder

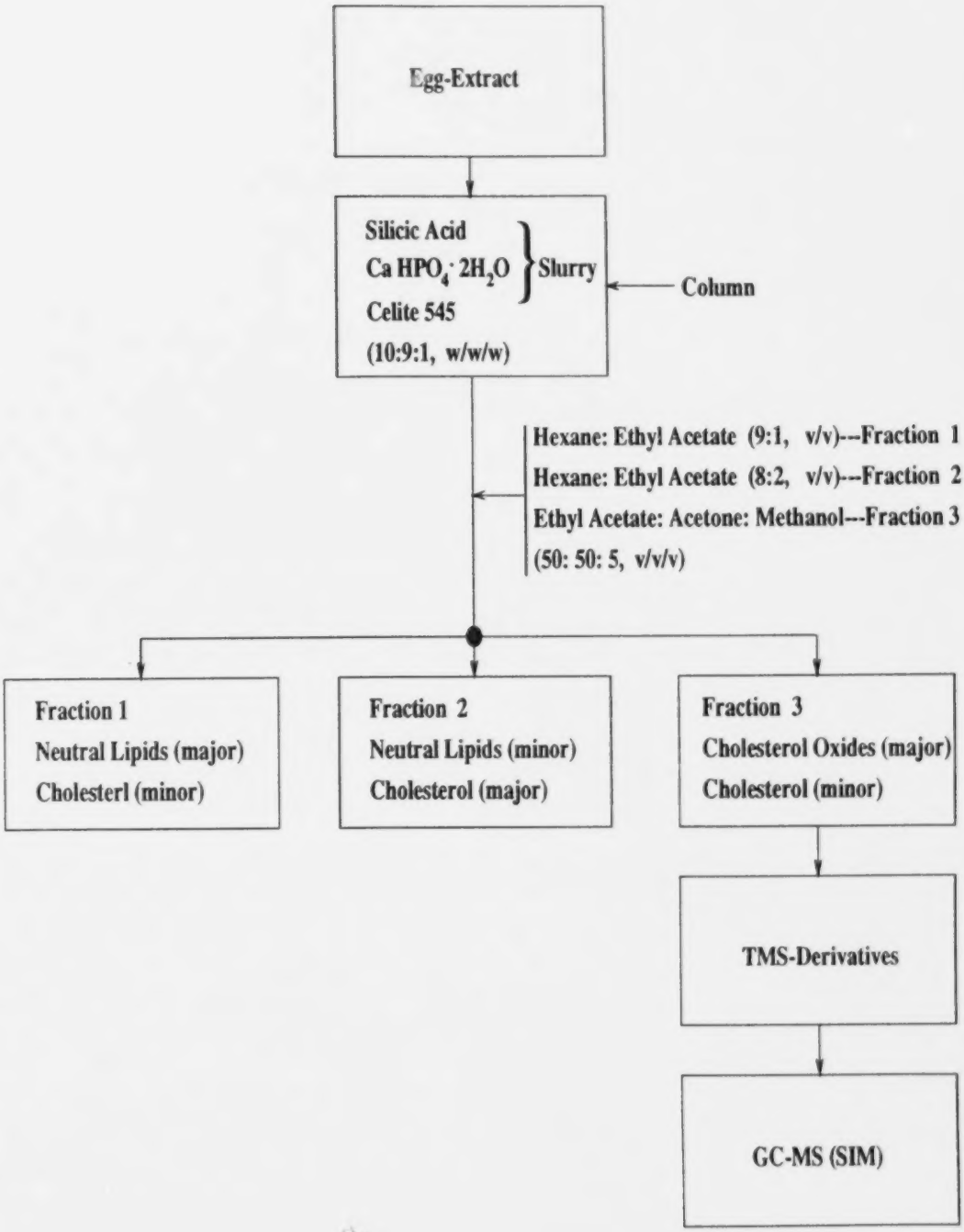

Egg-Extract
Silicic Acid
Ca HPO4 · 2H2O
Celite 545
(10:9:1, w/w/w)
Slurry
Column
Hexane: Ethyl Acetate (9:1, v/v)---Fraction 1
Hexane: Ethyl Acetate (8:2, v/v)---Fraction 2
Ethyl Acetate: Acetone: Methanol---Fraction 3
(50: 50: 5, v/v/v)
Fraction 1
Neutral Lipids (major)
Cholesterl (minor)
Fraction 2
Neutral Lipids (minor)
Cholesterol (major)
Fraction 3
Cholesterol Oxides (major)
Cholesterol (minor)
TMS-Derivatives
GC-MS (SIM)

Figure 3-2. SIM of TMS Derivatives of Synthetic Cholesterol Oxides

1. 5α-Cholestane
2. 7α-Hydroxycholesterol
3. 7β-Hydroxycholesterol
4. β-Epoxide
5. α-Epoxide
6. Cholestanetriol
7. 6-Ketocholesterol
8. 25-Hydroxycholesterol
9. 7-Ketocholesterol

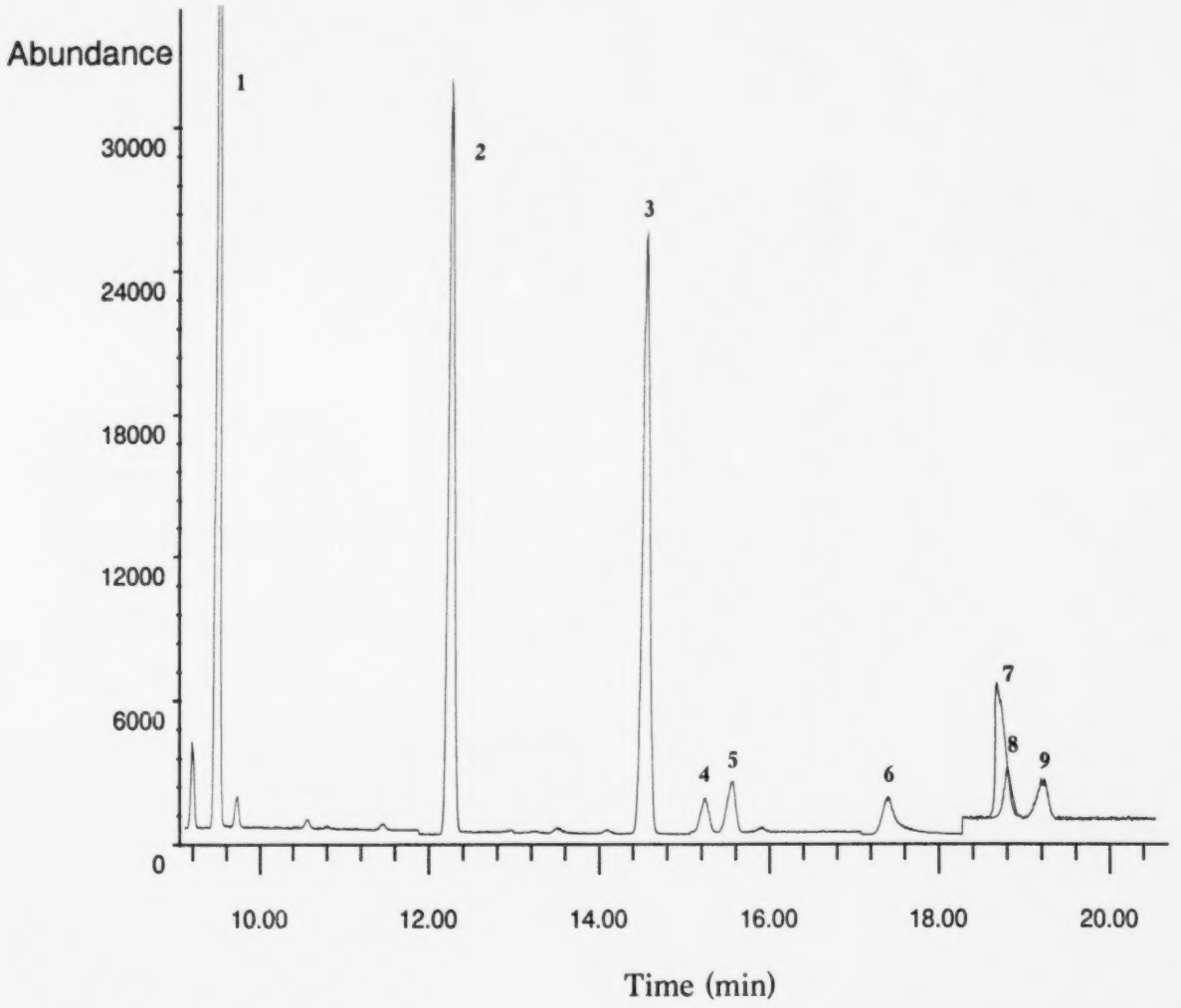

Abundance
30000
24000
18000
12000
6000
0
1
2
3
4
5
6
7
8
9
10.00
12.00
14.00
16.00
18.00
20.00
Time (min)

Figure 3-3. SIM of TMS Derivatives of Cholesterol Oxides Isolated from Spray Dried Egg Yolk Powder of Fish Oil Diet (FO) Stored at Room Temperature for Four Months

1. 5α-Cholestane
2. 7α-Hydroxycholesterol
3. 7β-Hydroxycholesterol
4. β-Epoxide
5. α-Epoxide
7. 6-Ketocholesterol
9. 7-Ketocholesterol

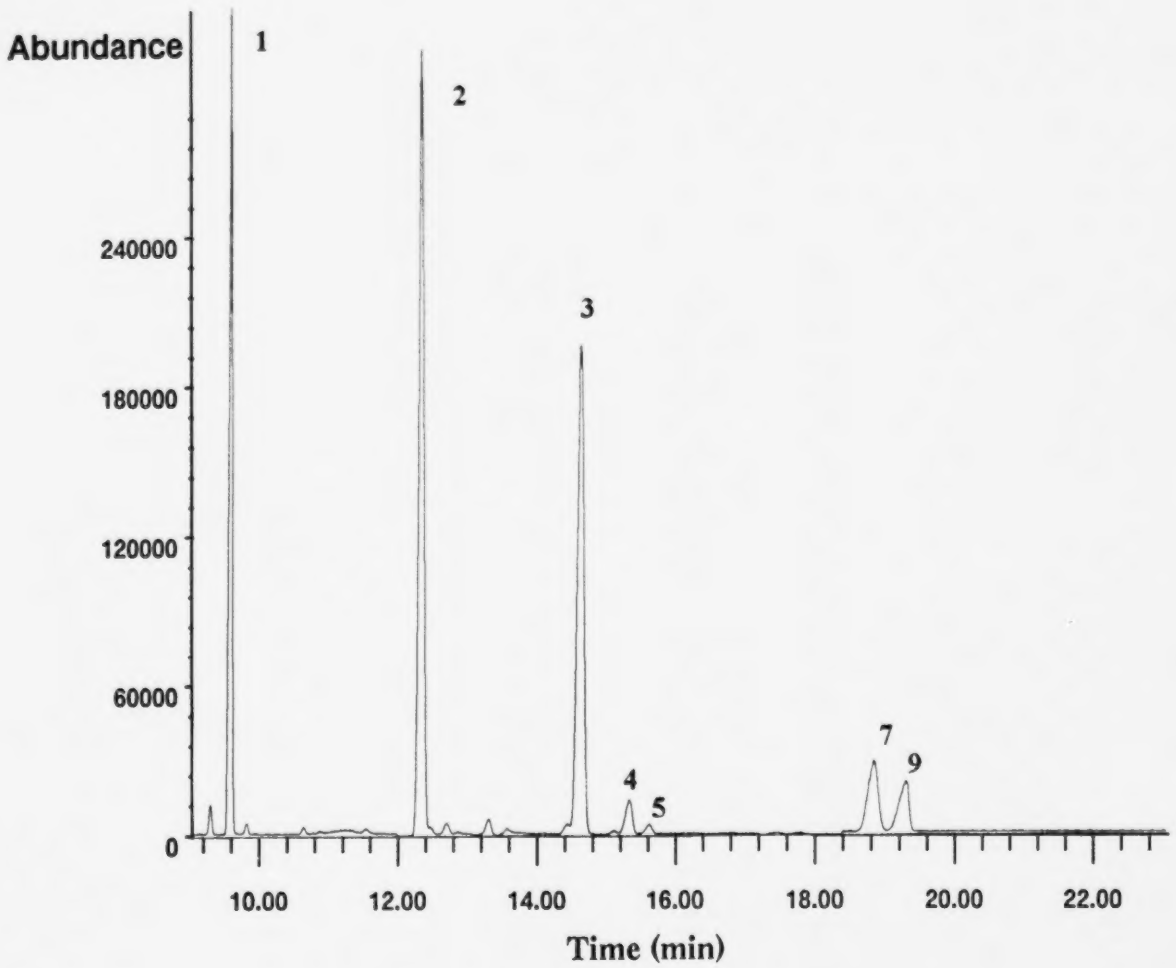

Abundance
1
2
3
4
5
7
9
240000
180000
120000
60000
0
10.00
12.00
14.00
16.00
18.00
20.00
22.00
Time (min)

Figure 3-4. Concentration of C-7 Oxidized Cholesterol Derivatives in Egg Yolk Powder with Different Dietary Oils and with and without Tocoherol Supplementation, Stored at Room Temperature for Four Months.

FO: fish oil diet.
LO: flax oil diet.
PO: palm oil diet.
SO: sunflower oil diet.
FO+T: fish oil + tocopherol diet.
LO+T: flax oil + tocopherol diet.
SO+T: sunflower oil + tocopherol diet.
PO+T: palm oil + tocopherol diet.
SEM was pooled: 2.11, 2.17, 2.94 for 7α-OH , 7β-OH and 7-Keto respectively.

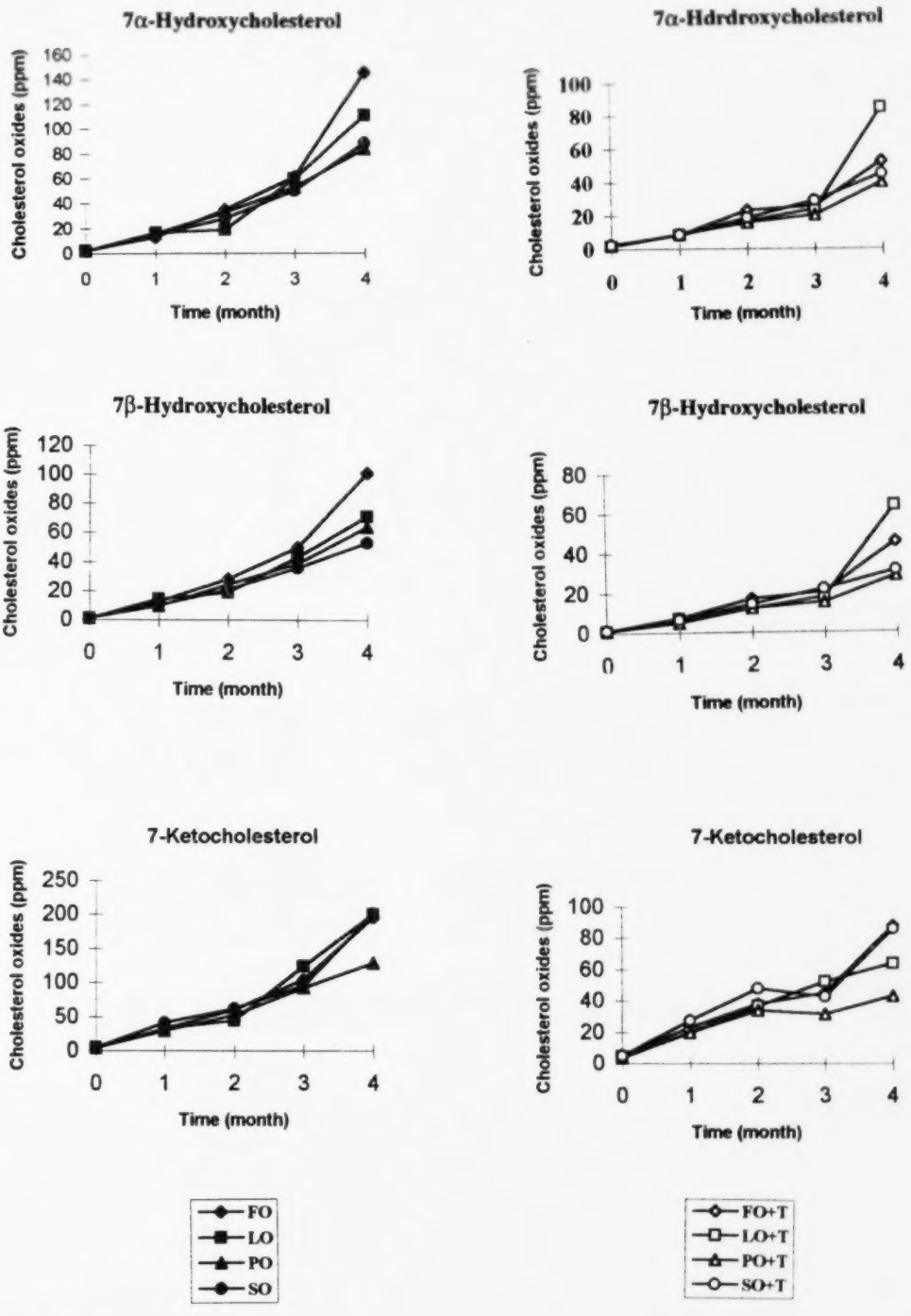
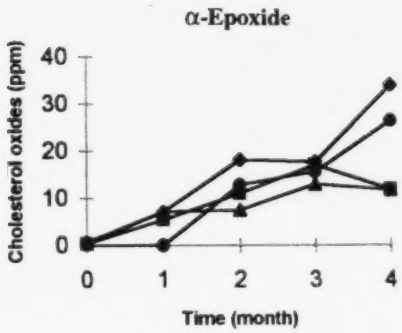
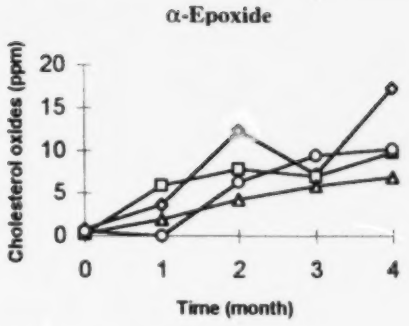
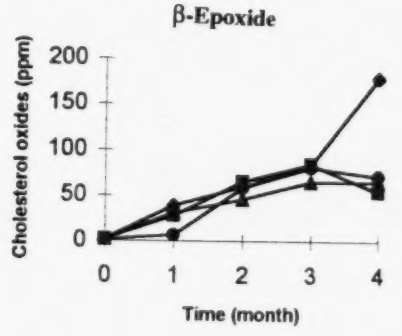

7α-Hydroxycholesterol
Cholesterol oxides (ppm)
Time (month)
7α-Hdrdroxycholesterol
Cholesterol oxides (ppm)
Time (month)
7β-Hydroxycholesterol
Cholesterol oxides (ppm)
Time (month)
7β-Hydroxycholesterol
Cholesterol oxides (ppm)
Time (month)
7-Ketocholesterol
Cholesterol oxides (ppm)
Time (month)
7-Ketocholesterol
Cholesterol oxides (ppm)
Time (month)
FO
LO
PO
SO
FO+T
LO+T
PO+T
SO+T

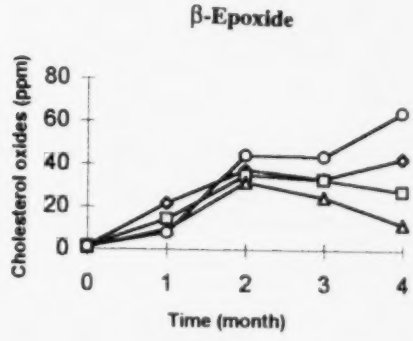
Figure 3-5. Concentration of α-Epoxde and β-Epoxide in Egg Yolk Powder with Different Dietary Oils and with and without Tocoherol Supplementation, Stored at Room Temperature for Four Months.

FO: fish oil diet.
LO: flax oil diet.
PO: palm oil diet.
SO: sunflower oil diet.
FO+T: fish oil + tocopherol diet.
LO+T: flax oil + tocopherol diet.
SO+T: sunflower oil + tocopherol diet.
PO+T: palm oil + tocopherol diet.
SEM was pooled: 0.94, 3.01 for α-Epoxde and β-Epoxide, respectively.


α-Epoxide
Cholesterol oxides (ppm)
40
30
20
10
0
0 1 2 3 4
Time (month)


α-Epoxide
Cholesterol oxides (ppm)
20
15
10
5
0
0 1 2 3 4
Time (month)


β-Epoxide
Cholesterol oxides (ppm)
200
150
100
50
0
0 1 2 3 4
Time (month)


β-Epoxide
Cholesterol oxides (ppm)
80
60
40
20
0
0 1 2 3 4
Time (month)

FO
LO
PO
SO

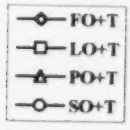

FO+T
LO+T
PO+T
SO+T

Table 3-1. Authentic Standards of Cholesterol and Cholesterol Oxides

	Sterol compounds	Common name
1	5α-Cholestane	5α-Cholestane
2	Cholest-5-en-3β, 7α-diol	7α-Hydroxycholesterol
3	Cholest-5-en-3β-ol	Cholesterol
4	Cholest-5-en-3β, 7β-diol	7β-Hydroxycholesterol
5	5,6α-Epoxy-5α-cholestan-3β-ol	α-Epoxide
6	5,6β-Epoxy-5β-cholestan-3β-ol	β-Epoxide
7	5α-Cholestane-3β, 5,6β-triol	Cholestane triol
8	Cholest-5-en-3β, 25-diol	25-Hydroxycholesterol
9	5α-Cholestan-3β-ol-6-one	6-Ketocholestanol
10	3β-Hydroxycholest-5-en-7-one	7-Ketocholesterol

Table 3-2. Percent Recovery of Cholesterol Oxidation Products from a Mixture of Pure Compounds and From Fresh Egg Yolk Powders[a]

Cholesterol oxidation product	Response factor (n=6)	Standard compounds 15ug each (n=6)	Added to egg yolkpowder 7.5ug/g (n=4)
7α-Hydroxycholesterol	0.18±0.02[b]	96.45 ± 10.71	104.12±4.66
7β-Hydroxycholsterol	0.23±0.01	96.23 ± 19.29	95.86 ±9.99
α-Epoxide	4.0±0.05	98.37 ± 7.55	97 ± 8.64
β-Epoxide	5.5±0.08	99.34 ± 7.02	101.57± 6.41
Cholestanetriol	3.5±0.05	86.13 ± 5.51	81.48 ±3.71
6-Ketocholestanol		99.66 ± 7.42	99.04 ± 3.9
7-Ketocholesterol	4.1±0.08	98.48 ± 9.12	109.67 ± 7.5

[a] Indicates addition of a known amount of standard to fresh egg yolk powder and quantification. The samples underwent entire extraction and glass column chromatography procedure prior to GC-MS (SIM).

[b] Mean ± SD

Table 3-3. Nutrient Composition of Laying Hen Diets

	Laying Hen Diets (%)							
Nutrients	FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T
Wheat	70.5	70.4	70.7	70.4	70.5	70.4	70.5	70.4
Soybean meal	14.5	14.5	14.4	14.5	14.5	14.5	14.5	14.5
Oil	3.5	3.5	3.4	3.5	3.5	3.5	3.5	3.5
Limestone	8.2	8.2	8.4	8.3	8.2	8.2	8.2	8.2
Calcium phosphate	2.3	2.3	2.0	2.2	2.3	2.3	2.3	2.3
Salt	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Methionine	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Layer premix[1]	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Tocopherol mix[2]		0.07		0.07		0.07		0.07
Calculated analyses								
CP, %	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0
ME, kcal/kg	2825	2823	2831	2824	2775	2772	2813	2810
Ether extracts, %	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9
Calcium, %	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6
Available P %	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Tocopherols (ppm)								
α	36.9	90.5	54.8	93.1	48.4	84.8	40.1	99.8
γ	3.0	164.3	27.1	211.3	7.4	207.1	1.9	200.5
δ	0.0	112.5	6.9	119.0	0.0	111.6	0.0	106.8
Total	39.9	367.4	88.8	423.4	55.7	403.5	47.2	407.1

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols; PO = Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil; SO+T = Sunflower oil + Tocopherols

SAFA = Saturated fatty acids; MUFA = Monounsaturated fatty acids; PUFA = Polyunsaturated fatty acids; P:S = Polyunsaturated : saturated fatty acid.

[1] Supplied per kilogram of the diet the following: vitamin A, 12,000 IU; vitamin D3, 3,000 ICU; vitamin E, 40 IU; riboflavin, 6.5 mg; calcium pantothenate, 14 mg; niacin, 40 mg; vitamin B12, 20μg; copper, 15 mg; selenium, 0.1 mg; iodine, 0.5 mg; biotin, 0.2 mg; manganese sulphate, 75 mg; zinc oxide, 80 mg; thiamine, 3.3 mg; folacin, 1 mg; Iron, 100 mg.

[2] The tocopherol mix contained 17.3, 36.3, 7.3, and 60.9% of δ, γ, α and total tocopherols.

Table 3-4. Fatty Acid Composition (%) of Laying Hen Diets

Fatty Acid	Diets							
	FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T
C14:0	5.5	5.7	0.0	0.0	0.7	0.7	0.1	0.1
C16:0	17.7	18.8	9.2	8.8	34.6	33.7	10.2	10.2
C18:0	2.7	2.7	2.3	2.5	3.5	3.6	3.9	3.9
C18:1	14.4	13.9	14.7	14.2	32.0	32.8	19.4	19.8
C18:2n6	22.7	20.4	31.0	28.7	25.9	26.2	62.5	60.0
C20:4n6	1.1	1.1	0.0	0.0	0.0	0.0	0.0	0.0
C18:3n3	2.7	2.7	43.6	45.3	1.9	1.9	2.1	2.1
C20:5n3	8.5	8.5	0.0	0.0	0.0	0.0	0.0	0.0
C22:6n3	7.4	7.8	0.0	0.0	0.0	0.0	0.0	0.0
SAFA	26.1	27.4	11.6	11.4	39.1	38.3	14.3	14.4
MUFA	14.4	13.9	14.7	14.2	32.0	32.8	19.4	19.8
PUFA	45.2	43.2	74.6	74.0	27.8	28.1	64.6	62.1
n6	24.6	22.1	31.0	28.7	25.9	26.2	62.2	60.0
n3	20.6	21.1	43.6	45.3	1.9	1.9	2.1	2.1
P:S	1.7	1.6	6.4	6.5	0.7	0.7	4.5	4.2

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols;
PO = Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil;
SO+T = Sunflower oil + Tocopherols
SAFA = Saturated fatty acids; MUFA = Monounsaturated fatty acids;
PUFA = Polyunsaturated fatty acids; P:S = Polyunsaturated : saturated fatty acid.

Table 3-5. Major Fatty Acid Composition of Egg Yolk Powder in Different Treatments before Storage

Fatty	Laying Hen Diets								
acid (%)	FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T	SEM
C16:0	26.1a	25.9a	22.7c	22.1c	24.9b	24.9b	24.7b	24.6b	0.24
C18:0	9.3c	9.7b	9.3c	9.3c	8.5d	8.8d	9.6b	10.2a	0.03
C16:1	4.2a	4.2a	3.5b	3.6b	3.5b	3.4b	2.7c	2.7c	0.05
C18:1	46.1b	44.2c	42.0d	44.5c	50.8a	50.8a	38.9f	39.5e	0.08
C18:2n-6	7.1f	7.8e	11.0c	10.2d	8.3e	8.3e	19.5a	18.4b	0.16
C20:4n-6	0.6e	0.6e	0.8d	0.8d	1.8c	1.9c	2.2a	2.1b	0.01
C18:3n-3	0.4b	0.4b	7.6a	7.0a	0.3b	0.2b	0.3b	0.3b	0.36
C20:5n-3	0.6a	0.7a	0.3b	0.3b	0.0	0.0	0.0	0.0	0.03
C22:6n-3	3.9a	3.7a	1.8b	1.7b	0.7c	0.6c	0.5c	0.7c	0.14
SAFA	36.0a	36.1a	32.3d	32.7d	33.8c	34.0c	34.6bc	35.2ab	0.29
MUFA	50.7b	48.7c	45.6e	47.8d	54.6a	54.5a	42.3g	42.7f	0.07
Total n-6	7.9g	8.6f	11.9c	11.2c	10.6e	10.5e	22.3a	21.0b	0.16
Total n-3	5.5d	6.7c	10.1a	9.2b	1.0e	0.9e	0.8e	1.1e	0.18
PUFA	13.3d	15.2c	22.0a	20.5b	11.6e	11.5e	23.1a	22.1a	0.34

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols;
PO = Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil; SO+T = Sunflower oil + Tocopherols
SAFA = Saturated fatty acids; MUFA = Monounsaturated fatty acids; PUFA = Polyunsaturated fatty acids
SEM = Standard error of mean.
a-g Means within same row with no common superscripts differ significantly ($P<0.05$)

Table 3-6. Major Fatty Acid Composition of Egg Yolk Powder from Different Treatments After Four Months of Storage

Fatty	Laying Hen Diets								
acid (%)	FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T	SEM
C16:0	27.1b	27.6a	23.9e	22.9g	25.9d	25.7de	26.4c	25.4e	0.1
C18:0	9.5c	9.7b	9.6b	9.3d	8.5f	8.7e	9.7b	10.3a	0.04
C16:1	4.5a	4.5a	3.6c	3.8b	3.6c	3.5d	2.9e	2.8f	0.03
C18:1	46.6b	44.7c	43.0e	44.3d	50.9a	51.0a	40.1f	39.7f	0.1
C18:2n-6	6.7f	7.0e	10.2b	9.9c	7.8d	7.9d	17.4a	17.5a	0.06
C20:4n-6	0.59d	0.49e	0.62d	0.67d	1.5c	1.6b	1.6b	1.8a	0.03
C18:3n-3	0.4d	0.4c	6.6a	6.5b	0.2e	0.2e	0.2e	0.2e	0.02
C20:5n-3	0.5b	0.6a	0.2d	0.2c	0.0	0.0	0.0	0.0	
C22:6n-3	2.7b	3.3a	1.2d	1.4c	0.5e	0.5e	0.3f	0.6e	0.03
SAFA	37.3b	38.0a	33.9f	32.5g	34.8e	34.7e	36.5c	36.1d	0.09
MUFA	51.3b	49.4c	46.8e	48.3d	54.7a	54.6a	43.4f	43.0g	0.09
PUFA	11.4e	12.6d	19.3c	19.2c	10.5f	10.7f	20.2b	20.9a	0.12
Total n-6	7.5e	7.7e	11.0c	10.8c	9.6d	9.9d	19.4b	19.8a	0.1
Total n-3	3.9d	4.9c	8.3b	8.5a	0.9f	0.9f	0.8f	1.12e	0.05

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols;
PO = Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil; SO+T = Sunflower oil + Tocopherols
SAFA = Saturated fatty acids; MUFA = Monounsaturated fatty acids; PUFA = Polyunsaturated fatty acids.
SEM = Standard error of mean.
a-g Means within same row with no common superscripts differ significantly ($P<0.05$)

Table 3-7. Changes of Total Tocopherol Content in Different Egg Yolk Powder during Storage

	Storage (month)					
Treatments[1]	0	1	2	3	4	Overall[2]
FO	$188.9^{g, v}$	$127.0^{g, w}$	$101.7^{f, x}$	$78.8^{f, y}$	$53.6^{f, z}$	109^{h}
FO+T	$687.5^{d, v}$	$558.5^{d, w}$	$565.6^{c, w}$	$541.4^{d, x}$	$448.9^{d, y}$	560^{d}
LO	$234.2^{f, v}$	$176.9^{e, w}$	$128.6^{de, x}$	$94.7^{e, y}$	$58.6^{ef, z}$	139^{f}
LO+T	$750.6^{c, v}$	$669.5^{c, w}$	$673.9^{b, w}$	$635.9^{c, x}$	$561.1^{c, y}$	658^{c}
PO	$250.7^{e, v}$	$189.5^{e, w}$	$133.6^{d, x}$	$98.9^{e, y}$	$74.1^{e, z}$	149^{e}
PO+T	$914.2^{a, v}$	$816.0^{b, w}$	$710.3^{a, x}$	$703.1^{a, x}$	$672.7^{a, y}$	763^{a}
SO	$228.0^{f, v}$	$154.7^{f, w}$	$117.8^{e, x}$	$79.1^{f, y}$	$38.8^{g, z}$	123^{g}
SO+T	$888.5^{b, v}$	$838.1^{a, w}$	$687.9^{b, x}$	$668.8^{b, y}$	$592.3^{b, z}$	735^{b}
Overall[3]	517.8^{v}	441.3^{w}	389.9^{x}	362.7^{y}	312.5^{z}	

FO = fish oil; FO+T = fish oil + α-tocopherol
LO = flax oil; LO+T = flax oil + α-tocopherol
SO = sunflower oil; SO+T = sunflower oil + α-tocopherol
PO = palm oil; PO+T = palm oil + α-tocopherol
a-h Means within same column with no common superscripts differ significantly ($P \leq 0.05$)
v-z Means within same row with no common superscripts differ significantly ($P \leq 0.05$)
[1] Standard error of mean (SEM) for 8 treatments during storage was pooled : 5.57
[2] Standard error of mean (SEM) for the overall was pooled: 2.49
[3]Standard error of mean (SEM) for the overall was pooled: 8.69

Table 3-8. Changes of Total Tocopherol Content in Different Egg Yolk Powder during heating

	Laying Hen Diets							
	FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T
	(μg/g yolk powder)							
Uncooked powder	188.9a	687.5a	234.2a	750.6a	250.7a	914.2a	228.0a	888.5a
Cooked powder	138.1b	567.9b	203.7b	649.5b	228.7b	754.5b	181.6b	706.4b

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols; PO = Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil; SO+T = Sunflower oil + Tocopherols
Pooled standard error of mean = 4.08
a-b Means within same column with no common superscripts differ significantly ($P<0.05$)

Table 3-9. The Changes of Lipid Classes in Egg Yolk Powder During Storage

Storage (month)	Laying Hen Diets								
	FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T	SEM
	Neutral lipids[1] (%)								
0	$67.5^{a, x}$	$70.1^{a, x}$	$60.1^{b, x}$	$63.9^{ab, x}$	$63.5^{ab, x}$	$65.5^{ab, x}$	$65.6^{ab, x}$	$67.9^{a, x}$	2.48
4	$48.9^{de, y}$	$44.4^{e, y}$	$53.0^{cd, y}$	$55.1^{bcd, y}$	$57.7^{abc, x}$	$62.4^{a, x}$	$56.5^{abc, y}$	$61.5^{ab, x}$	
	Polar lipids (%)								
0	$32.2^{b, y}$	$29.7^{b, y}$	$39.9^{a, y}$	$36.1^{ab, y}$	$36.5^{ab, x}$	$34.6^{ab, x}$	$34.4^{ab, y}$	$32.1^{b, x}$	2.44
4	$51.1^{ab, x}$	$55.9^{a, x}$	$47.0^{bc, x}$	$45.1^{bcd, x}$	$41.6^{cde, x}$	$37.6^{e, x}$	$43.5^{cde, x}$	$38.5^{de, x}$	

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols;
PO = Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil; SO+T = Sunflower oil + Tocopherols
SAFA = Saturated fatty acids; MUFA = Monounsaturated fatty acids; PUFA = Polyunsaturated fatty acids.
[1] Neutral lipids including triglycerides, diglycerides and cholesterol
SEM = Standard error of mean.
a-e Means within same row with no common superscripts differ significantly ($P<0.05$)
x-y Means within same column with no common superscripts differ significantly ($P<0.05$)

Table 3-10. Changes of Lipid Classes of Egg Yolk Powder between Unheated vs Heated Treatments

	Laying Hen Diets								
	FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T	SEM
	Neutral lipids[1] (%)								
Uncooked powder	67.4[a]	70.0[a]	60.0[b]	64.0[a]	63.4[b]	65.5[a]	65.6[a]	67.9[a]	2.7
Cooked powder	73.6[a]	72.8[a]	70.2[a]	59.4[a]	72.6[a]	71.2[a]	67.2[a]	68.2[a]	
	Polar lipids (%)								
Uncooked powder	32.2[a]	29.7[a]	40.0[a]	36.1[a]	36.5[a]	34.6[a]	34.4[a]	32.1[a]	2.73
Cooked powder	26.3[a]	27.3[a]	31.8[b]	40.8[a]	27.4[b]	28.8[a]	32.8[a]	31.8[a]	

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols;
PO = Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil; SO+T = Sunflower oil + Tocopherols
SAFA = Saturated fatty acids; MUFA = Monounsaturated fatty acids; PUFA = Polyunsaturated fatty acids.
[1] Neutral lipids including triglycerides, diglycerides and cholesterol
SEM = Standard error of mean.
a-b Means within same row with no common superscripts differ significantly ($P< 0.05$)

Table 3-11. Effects of Dietary Oils and Tocopherools and Storage on the Total Cholesterol oxidation in the Egg Yolk Powder

	Storage (month)					
Treatmet[1]	0	1	2	3	4	[2]overall
FO	$9.86^{a,z}$	$103.4^{a,y}$	$200.4^{a,x}$	$315.6^{a,w}$	$656.2^{a,v}$	257.1^{a}
FO+T	$11.4^{a,y}$	$63.7^{bc,x}$	$127.8^{d,w}$	$131.4^{c,w}$	$246.7^{d,v}$	116.2^{e}
LO	$10.8^{a,z}$	$96.1^{a,y}$	$158^{c,x}$	$328.7^{a,w}$	$448.4^{b,v}$	208.4^{b}
LO+T	$8.4^{a,z}$	$54.9^{bc,y}$	$107.9^{e,x}$	$133.5^{c,w}$	$249.9^{d,v}$	110.9^{e}
PO	$9.12^{a,z}$	$92.3^{a,y}$	$162^{c,x}$	$261.8^{b,w}$	$352.4^{c,v}$	175.5^{d}
PO+T	$6.98^{a,y}$	$44.3^{d,x}$	$97.3^{e,w}$	$97.1^{d,w}$	$130.7^{e,v}$	75.3^{f}
SO	$9.08^{a,z}$	$72.1^{b,y}$	$181.6^{b,x}$	$277.6^{b,w}$	$439.9^{b,v}$	196.1^{c}
SO+T	$9.6^{a,y}$	$50.4^{cd,x}$	$130.9^{d,w}$	$146.5^{c,w}$	$237.1^{d,v}$	114.9^{e}
Overall[3]	9.4^{z}	72.2^{y}	145.7^{x}	211.5^{w}	345.2^{v}	

FO = fish oil; FO+T = fish oil + α-tocopherol
LO = flax oil; LO+T = flax oil + α-tocopherol
SO = sunflower oil; SO+T = sunflower oil + α-tocopherol
PO = palm oil; PO+T = palm oil + α-tocopherol
a-f Means within same column with no common superscripts differ significantly ($P<0.05$)
v-z Means within same row with no common superscripts differ significantly ($P<0.05$)
[1] Standard error of mean (SEM) for 8 treatments during storage was pooled : 6.52.
[2] Standard error of mean (SEM) for the overall was pooled: 2.92
[3] Standard error of mean (SEM) for the overall was pooled: 2.31

Table 3-12. Effects of Heating and Tocopherols on Cholesterol Oxidation in Different Egg Yolk Powder

Diets	Total COPs	Major COPs* (ug/g) 7α-OH	7β-OH	α-Epoxide	β-epoxide	7-keton	25-OH
			Unheated yolk powder				
FO	9.8	2.1	1.2	0.70	2.1	3.6	nd
FO+T	11.3	2.5	1.1	0.80	1.4	5.5	nd
LO	10.3	2.2	1.2	nd	1.8	5.1	nd
LO+T	8.0	1.6	0.8	nd	1.3	4.3	nd
PO	8.6	2.0	1.1	nd	1.4	4.1	nd
PO+T	5.5	1.8	0.73	nd	0.7	2.4	nd
SO	8.7	2.2	1.0	nd	1.2	4.3	nd
SO+T	9.0	2.0	1.1	nd	1.1	4.8	nd
SEM	0.19	0.04	0.05		0.06	0.12	
			Heated yolk powder				
FO	296.2^{a}	39.5^{a}	48.6^{a}	14.1^{a}	98.2^{a}	89.3^{a}	6.54
FO+T	225.4^{bc}	30.4^{c}	36.6^{c}	13.1^{a}	78.5^{b}	62.8^{c}	3.96
LO	246.1^{b}	34.4^{b}	42.5^{b}	11.9^{ab}	83.1^{b}	71.0^{b}	3.11
LO+T	190.2^{de}	26.5^{d}	31.5^{d}	9.8^{b}	66.6^{c}	55.8^{cd}	nd
PO	179.5^{e}	25.8^{de}	30.3^{d}	8.9^{b}	64.7^{c}	49.8^{d}	nd
PO+T	151.0^{f}	22.5^{e}	26.3^{e}	8.8^{b}	51.9^{d}	41.5^{e}	nd
SO	200.8^{cd}	27.8^{cd}	31.1^{d}	9.4^{b}	80.4^{b}	52.1^{d}	nd
SO+T	203.3^{cd}	27.2^{cd}	30.3^{d}	11.3^{ab}	82.3^{b}	52.3^{d}	nd
SEM	7.47	1.18	1.28	1.05	3.98	2.6	nd

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols; PO= Palm oil; PO+T = Palm oil +Tocopherols; SO = Sunflower oil; SO+T = Sunflower oil +Tocopherols.
7α-OH: 7α-Hydroxycholesterol; 7β-OH: 7β-Hydroxycholesterol; 7-Keto: 7-Ketocholesterol; 25-OH: 25 hydroxycholesterol;
* COPs = Cholesterol oxidation products
nd = nondetected
SEM = Standard error of mean
a-f Means within same column with no common superscripts differ significantly ($P<0.05$)

Table 3-13. Effects of Dietary Oils and Tocopherols and Storage on the Loss of Cholesterol and Formation of its Oxides

Sterols Content	Storage (month)	FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T
Cholesterol (mg/g of yolk)	0	23.7±0.4*	21.7±0.3	21.3±0.1	21.0±0.2	22.3±0.5	20.5±1.3	20.7±0.3	20.5±0.7
	4	10.3±0.1	10.3±0.2	9.7±0.2	9.2±1.5	10.0±0.2	10.2±0.2	9.2±0.2	10.0±0.1
Total[1] COPs (ppm)	4	656.2±21	246.7±10	448.4±10	249.9±24	352.4±30	130.7±5	439.9±22	237.1±10
Total COPs /cholesterol %	4	2.8	1.1	2.1	1.2	1.6	0.6	2.1	1.2

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols;
PO = Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil; SO+T = Sunflower oil + Tocopherols.
[1] Total cholesterol oxides
* Mean ± SD (n=3)

Table 3-14. Effects of Dietary Oils and Tocopherols and Heating on the Loss of Cholesterol and Formation of its Oxides

Sterols Content		FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T
Cholesterol (mg/g of yolk)	Uncooked powder	23.7±0.4*	21.5±0.3	21.3±0.1	21.0±0.2	21.3±0.5	20.5±1.3	20.7±0.3	20.5±0.7
	Cooked powder	23.1±0.8	21.1±0.4	20.8±0.2	20.6±0.14	20.8±0.3	20.2±0.7	20.1±0.4	19.9±0.1
Total[1] COPs (ppm)	Cooked powder	296.2±13	225.4±11	246.1±6	190.2±16	179.5±20	151.0±6	200.8±18	203.3±9
Total COPs /cholesterol %	Cooked powder	1.25	1.04	1.15	0.91	0.84	0.74	0.97	0.99

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols;
PO = Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil; SO+T = Sunflower oil + Tocopherols.
[1] Total cholesterol oxides
* Mean ± SD (n=3)

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Chapter 4. Effects of Dietary oil and Tocopherol Supplementation on Lipid and Cholesterol Oxidation in Chicken Meat

4. 1. Introduction

Cholesterol is widely distributed in foods, of which animal fats, meats and egg products contain significant amounts. In North America, animal fat normally accounts for approximately 57% of total fat available for consumption. In Canada, the average intake of cholesterol is about 440 mg per day per person (Health and Welfare Canada, 1990). The association between cholesterol and cardiovascular disease has been recognized for many years. However, the results of many cholesterol feeding studies have been questioned following some observations that a mixture of oxidation products of cholesterol was atherogenic to rabbits and chickens (Imai et al., 1976; Taylor et al., 1979; Toda et al., 1982).

Cholesterol is an unsaturated compound and is readily autoxidized to form cholesterol oxidation products. Their presence in processed food has attracted much concern since cholesterol oxides have been detected in several food ingredients such as raw, cooked, and dehydrated meats (Sander et al., 1989), dairy products (Cleveland and Harris, 1987), fish products (Ohshima et al., 1993; Li et al., 1994) and egg products (Naber and Biggert, 1985; Addis, 1986). Accordingly, the scientific study of autoxidation of cholesterol in food has become a critical research needs. The ingestion of COPs may

have implications for human health, particularly in the long term, and detrimental effects on the blood-vascular system.

The influence of diet on the fatty acid composition of animal tissue, particularly muscle and adipose tissue, has been the subject of much investigation (Villegas et al., 1973; Westerling and Hedrick, 1979; Larick and Turner, 1989; Ajuyah et al., 1993). Modification of the fatty acid composition of livestock and fowl by dietary means is receiving increasing attention, not only because of the probable relationship between fatty acid intake, plasma cholesterol and heart disease (Grundy, 1989), but also because of the effect of the fatty acid profiles on the oxidative stability of animal tissues (Hertzman et al., 1988; Ajuyah et al., 1993). Relative to red meat, consumption of meats with higher levels of polyunsaturated fatty acids such as poultry and fish has increased in recent years, and this trend is likely to continue. Furthermore, there is an increasing trend towards the production of convenience foods using comminuted poultry meat (Chen and Waimaleongora-Ek, 1981). Enrichment of chicken meat with omega-3 fatty acids by incorporating flax and canola seeds or their oils in laying hen diets has been reported (Ajuyah et al., 1991a, 1991b). The substantial amounts of omega-3 fatty acids in poultry products decrease the saturation and could enhance lipid oxidation. The susceptibility of muscle tissue to lipid oxidation depends on a number of factors, the most important being the level of polyunsaturated fatty acids present in the particular muscle system (Allen and Foegeding, 1981). Oxidation of unsaturated fatty acids proceeded through a free-radical chain mechanism involving initiation, propagation, and termination steps. Smith(1980) indicated that cholesterol autoxidation was a free radical mechanism, similar to

autoxidation of unsaturated fatty acids (Nawar, 1985). Ohshima et al. (1993) had reported that cholesterol oxidation in fish products proceeds in conjunction with oxidation decomposition of the coexisting polyunsaturated fatty acids of fish oils. The previous chapter has demonstrated that the presence of long chain polyunsaturated fatty acids accelerated cholesterol oxidation in egg yolk powder and food oils.

The susceptibility of muscle to lipid oxidation is also influenced by the presence of antioxidants. It has long been recognized that α-tocopherol can function as an antioxidant in tissues (Olcott and Mattill, 1941) by inhibiting the oxidative reactions of membrane-bound lipids caused by free radical attack. The phenolic group of α-tocopherol acts as an electron donor, deactivating the catalysts, as well as suppressing the formation of lipid peroxy radicals, and thus preventing propagation of chain reaction (Asghar et al., 1990). Dietary vitamin E supplementation has been shown to improve the oxidative stability of fish muscle (Frigg et al., 1990), chicken (Asghar et al., 1990) and pork (Monahan et al., 1992). In laying hens, supplemental vitamin E reduced subsequent TBA values of gastrocnemius muscle under frozen storage (Combs and Regenstein, 1980). It has been reported that α-tocopherol supplementation of poultry diets results in an increase in the stability of the meat (Sklan et al., 1983; Sheldon, 1984; Lin et al., 1989; Asghar et al., 1990). The objective of this study was to investigate the effects of dietary oils and tocopherol supplementation on lipid and cholesterol oxidation of muscle during storage.

4. 2. Materials and Methods

Reagents

Cholesterol and cholesterol oxide standards (> 99% purity), including 7α-hydroxycholesterol, 7β-hydroxycholesterol, α-epoxide, β-epoxide, cholestanetriol, 7-ketocholesterol and 6-ketocholestanol, were purchased from Sigma Chemical Co. (St Louis, MO) and Steraloids Inc. (Bayonne, New Hampshire). 5α-cholestane, Butylated hydroxyltoulene (BHT), 2-thiobarbituric acid (TBA) and tocopherol isomer standards were purchased from Sigma Chemical Co. (St Louis, MO). All standards were used as purchased. Celite 545 and Calcium phosphate ($CaHPO_4 \bullet 2H_2O$) were purchased from Fisher Scientific Co. (Malvern PA) and silicic acid (100 mash) from Aldrich Chemical Co. (Milwaukee, WI). Acetonitrile and methanol were HPLC grade from BDH Inc., (Toronto, Ontario) and all other solvents used were glass distilled.

Experimental Design

Single Comb White Leghorn pullets, 22 wk of age, were housed in two double-deck cage batteries with two birds in each cage (.31 x .40 m). Each battery had 120 cages and divided into 8 units with 15 cages per unit, thus generating a total of 16 experimental units. The birds were allotted to eight dietary treatments. The experimental diets were as described in Chapter 3 (see Table 3-3 for the laying hen diets). Six hens were randomly selected and killed by cervical dislocation at the end of feeding test diet. Tissue samples from breast meat were excised. Each treatment was randomly separated into three groups (two chicken meat samples per group) and weighed, then freeze dried (Model 50-SRC.

Virtis company, Virtis Gardiner, N. Y. 12525), and ground into powder with a Hobart meat grinder. Triplicate meat powder samples from each of eight treatments were stored in polythene container at room temperature and open to air. The samples were shaken daily to ensure uniformity of light (normal daylight cycle) and air access. Small aliquots were taken from each of the triplicate samples of eight batches of powder and analyzed at 0 and 4 months of storage.

Fatty Acid Composition Analyses

Total lipids were extracted from meat powder by the method of Folch et al. (1957). Aliquots of the lipid extracts were dried under nitrogen and were converted to fatty acid methyl esters using a mixture of boron trifluoride, hexane and methanol (35:20:45, v/v/v) (Metcalfe and Schmitz, 1961). Fatty acid methyl esters were separated and quantified by gas chromatography as described in Chapter 2.

Cholesterol Assay

About 0.5 g of freeze-dried meat powder samples were saponified according to Fenton and Sim (1991). The unsaponifiable fraction was extracted using 10 ml of hexane, and the hexane extracts were assayed for cholesterol by gas chromatography (GC) described in Chapter 3. Two mg of 5α-cholestane (Sigma Chemical Co., St. Louis, MO 63178) was added to each meat powder sample as internal standard (IS) before saponification.

Tocopherol Assay

Tocopherols were extracted according to Zaspel et al. (1983), with minor modification. The freeze-dried meat samples (ca. 500 mg) were weighed in duplicate. Two hundred µl of the internal standard (rac-5,7-dimethyltocol, 10 ug/ml in Methanol) and 5 ul of BHA (1 mg/ml in ethanol) were added, and homogenized in 8 ml of acetone using a Brinkman Polytron (Type PT 10/35, Brinkman Instruments Inc., Westbury, NY. 11590-0207) for 20 s at top speed. Another 3 ml of acetone were used to rinse the probe. The homogenate was centrifuged at 2000 rpm in a Beckman centrifuge (Beckman Instruments, Mississauga, Ontario, Canada) for 10 min. and the supernatant was transferred to a test tube. The pellet was reextracted with 4 ml of acetone. All of the supernatant fractions were pooled and dried under N_2 at 30-40°C and redissolved with 2 ml of methanol. α, γ and δ-tocopherol concentrations in the meat powder samples were determined using HPLC as described in Chaper 3.

2-Thiobarbituric Acid (TBA) Analyses

2-thiobarbituric acid numbers were determined on freeze-dried chicken meats on day 0, and after 4 months as described by Salih et al. (1987), with some modifications. Two g of meat samples were weighed into 50 ml test tubes, and 18 ml of 3.86% perchloric acid added. The sample was homogenized with a Brinkman Polytron (Type PT 10/35, Westbury, NY) for 15 s at high speed (set at 8-9). BHT was added (125 mg/g fat) to each sample to control lipid autoxidation. The homogenate was filtered through Whatman #1 filter paper. Filtrate (2 ml) was mixed with 2 ml of 20 mM TBA in distilled

water, and incubated at 100°C for 20 min. Absorbance was determined at 531 nm against a blank containing 2 ml distilled water and 2 ml of 20 mM TBA solution. The TBA numbers were expressed as mg malonaldehyde/kg meat.

Iatroscan TLC-FID of Neutral and Polar Lipids

Lipids were extracted using the method of Folch et al. (1957). An Iatroscan TH-10 TLC Analyzer MK II (#93804 Iatron Laboratories, Tokyo, Japan) equipped with a flame ionization detector was used to quantify the neutral and polar lipid classes in the meat powders as described in Chapter 3.

Cholesterol Oxidation Products Analyses

Total lipids were extracted by the method of Folch et al. (1957). A column chromatography method for isolation of cholesterol oxides and cholesterol oxides were determined qualitatively and quantitatively by gas chromatography as previously described in Chapter 3.

Statistical Analyses

One-way ANOVA was used to analyze the differences in fatty acids among different diet within storage. Data for cholesterol oxides, and tocopherols were analyzed using two-way analyses of variance with sources of variation of oils (o=4), tocopherol (t=2) within storage. TBA and lipid classes were analyzed using three-way analyses of variance with sources of variation of oil (o=4), tocopherol (t=2), and storage (s=2). Means

of effects (oil, tocopherol, storage) were separated by the Student-Newman-Keuls (SNK) test (Steel and Torrie, 1980). Means of interaction were compared using the PDIFF function of the SAS program (SAS Institute, 1985).

4. 3. Results and Discussion

The major fatty acid composition of the diets are shown in Table 3-4 (Chapter 3). The levels of different fatty acids in different oil based diets were proportional to levels of dietary incorporation of each oil (Table 3-4). Fatty acid profiles of eight treatments are presented in Table 4-1 and Table 4-2. The fatty acid composition of breast muscle were significantly modified by the laying hen diets (Table 4-1). Linolenic acid (LNA, C18:3n3) was the major n-3 PUFA deposited in flax oil diets (7.2-7.9%). Long chain polyunsaturated fatty acids, such as C20:5n3 and C22:6n3, were deposited in meat of fish oil diets, 0.57-0.87 and 1.9-3.6%, respectively. Meat from the sunflower oil diet mainly contained n-6 linoleic acid (LA, C18:2n6), 18.2-19.5%. Conversely, the palm oil diet resulted in a significant decrease of total n-3 , n-6 and PUFA and a higher total saturated fatty acids (SAFA) content and oleic acid (C18:1n9) in meats. Total omega-3 and omega-6 and PUFA was decreased concomitantly with an increase in total SAFA in all the treatments after four month storage (compare Table 4-1 and Table 4-2).

Mean tocopherol concentrations in meats were significantly ($P<0.05$) influenced by diet (Table 4-3). In hens receiving the tocopherol-supplemented diet, tocopherol concentrations were approximately 2-times higher in meats than for non-supplemented

diets. One possible explanation is that the additional vitamin E was absorbed by the animals and incorporated into cellular membranes where it performed its antioxidant function (Faustman et al., 1989). Dietary oil had no apparent influence on the deposition of tocopherols in the breast muscle. After 4 months of storage, all treatments had no detectable tocopherols.

The TBA values for freeze-dried meat powders are presented in Table 4-4. TBA values increased significantly ($P<0.05$) with time of storage except in fish oil without tocopherols treatment (FO). However, no significant difference was observed between tocopherol supplemented and non-supplemented sunflower, flax and palm oil treatments after 4 months of storage. The fish oil diet was the exception. Fish oil without tocopherol treatment (FO) had high initial TBA numbers, and TBA numbers did not change after 4 months of storage. This may be due to fish oil being easily oxidized and having high TBA value after freeze drying. Thus, this method might not be used for analyzing the higher TBA values. Moreover, Evaluating lipid oxidation by TBA alone may not a wise way. Total tocopherols in all treatments disappeared after four months of storage. The protective effect of tocopherols decreased such that TBA values increased in all treatments.

Data related to neutral and polar lipid content of freeze dried chicken meat powders stored at room temperature for up to four months are shown in Table 4-5. After four months of storage, polar lipids significantly ($P<0.05$) increased concomitantly with a decrease in neutral lipids. These results show that change of neutral and polar lipid ratio may indicate quality of food during storage. Both polymerized products and polar material

may prove quite useful for assessing deterioration of heated oils (Hara et al., 1989).

Cholesterol oxidation products of freeze dried meat powders are presented in Table 4-6. Initial levels of cholesterol oxides before storage were 2.8-5.5 ppm and were apparently increased (154-259 ppm) after four months of storage. Fish oil and sunflower oil without tocopherols (FO and SO) had higher levels of oxides (257 and 260 ppm respectively), followed by a flax oil diet (224 ppm). The palm oil (PO) diet had a low content of oxides (189 ppm). The difference in susceptibility of cholesterol oxidation between the fish oil diet and other vegetable oil diets may be due to different degrees of unsaturation. This is supported by the fact that low levels of cholesterol oxides accumulated in palm oil diets containing high saturated fatty acids and C18:1n9. This suggests that LCPUFA (C20:5n3 and C22:6n3) greatly influences the cholesterol oxidation. Li et al (1994) demonstrated that sardine oil triacylglycerols with cholesterol contained 920 ppm of oxides while fully hydrogenated triacylglycerols with cholesterol had no oxide content. Cholesterol oxides contents were significantly ($P<0.05$) lower in tocopherol supplemented treatments than in treatments without tocopherol during storage. Similarly, palm oil treatment with tocopherol had the lowest level of oxides (154 ppm) even though no significant differences were observed between the tocopherol supplemented and non-supplemented palm oil diets (PO and PO+T). These results agree with previous chapters.

Overall, the average of all the products indicates the highest levels of 7-ketocholesterol, followed by β-epoxide, 7α-hydroxycholesterol, 7β-hydroxycholesterol and α-epoxide. 7-ketocholesterol has been reported as a principal cholesterol oxide product

(Maerker, 1987) and has been used as a marker for cholesterol oxidation in ground beef (De Vore, 1988) and in aqueous model systems (Rankin and Pike, 1993). Fontana et al. (1993) also reported that β-epoxide was a predominant derivative with aged egg powder and 7-ketocholesterol was predominant in heated egg powder. The present results show the presence of cholestanetriol, the more toxic derivative of cholesterol (Imai et al., 1976; Blankeship et al., 1991) in all treatments. This strongly suggests the need for investigations on cholesterol products in commercial dehydrated meat products. Effects of dietary oils, tocopherols, and storage on the loss of cholesterol and formation of its oxides are shown in Table 4-7. Ca 50 % cholesterol was lost, compared to initial contents of samples in storage. The present study shows that the amount of cholesterol oxides can reach 11-18.4% of total cholesterol during storage. A major portion of cholesterol decomposition may have occurred via a different pathway, e.g., polymerization (Nawar et al., 1991).

In summary, cholesterol oxide levels in chicken meat were affected by daylight and storage. Fatty acid composition and tocopherol content in powders influenced the stability of cholesterol. Meat powder from laying hen's fed fish oil was easily oxidized. Cholesterol oxidation is accelerated by the presence of highly long chain polyunsaturated fatty acids. Tocopherol supplementation in hen's diets improved oxidative stability of cholesterol. This indicates the potential for a supplementation program in food-producing animals in order to produce higher-quality meat and meat products and to protect the consumer from the potentially deleterious biological effects of lipid oxidation products.

Table 4-1. Major Fatty Acid Composition of Freeze-Dried Chicken Meat in Different Treatments before Storage

Fatty	Laying Hen Diets								
acid (%)	FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T	SEM
C16:0	24.2^{b}	25.8^{a}	22.3^{c}	21.7^{c}	25.6^{a}	25.4^{a}	24.0^{b}	24.0^{b}	0.37
C18:0	5.9^{c}	7.6^{a}	6.9^{b}	6.6^{b}	6.8^{b}	6.5^{bc}	8.1^{a}	7.6^{a}	0.22
C16:1	6.6^{a}	5.2^{b}	4.0^{d}	4.3^{cd}	4.8^{bc}	4.8^{bc}	3.4^{e}	4.2^{cd}	0.19
C18:1	42.7^{a}	37.6^{bc}	37.0^{bc}	38.1^{b}	41.8^{a}	42.5^{a}	34.8^{c}	36.9^{bc}	1.04
C18:2n6	12.8^{e}	12.3^{e}	16.3^{bc}	15.7^{cd}	13.5^{de}	13.7^{de}	19.5^{a}	18.2^{ab}	0.81
C20:4n6	1.7^{e}	3.2^{cd}	2.5^{de}	2.3^{e}	3.6^{c}	3.2^{cd}	5.4^{a}	4.5^{b}	0.27
C18:3n3	0.9^{c}	0.9^{c}	7.2^{b}	7.9^{a}	0.7^{c}	0.7^{c}	0.7^{c}	0.9^{c}	0.16
C20:5n3	0.5^{b}	0.8^{a}	0.2^{c}	0.2^{c}	0.0^{d}	0.0^{d}	0.0^{d}	0.0^{d}	0.03
C22:5n3	0.4^{c}	0.8^{a}	0.6^{b}	0.5^{bc}	0.2^{d}	0.2^{d}	0.3^{d}	0.3^{d}	0.04
C22:6n3	1.9^{b}	3.6^{a}	1.0^{c}	0.9^{cd}	0.7^{de}	0.6^{e}	0.9^{cde}	0.8^{cde}	0.1
SAFA	31.5^{c}	34.7^{a}	29.9^{d}	28.9^{d}	33.2^{ab}	32.7^{bc}	32.8^{bc}	32.3^{bc}	0.55
MUFA	49.6^{a}	43.0^{b}	41.3^{bc}	42.6^{b}	46.8^{a}	47.5^{a}	38.4^{c}	41.4^{bc}	1.18
PUFA	18.8^{c}	22.3^{b}	28.8^{a}	28.4^{a}	20.0^{bc}	19.8^{bc}	28.8^{a}	26.4^{a}	1.04
Total n6	14.9^{d}	16.1^{cd}	19.6^{b}	18.7^{bc}	18.0^{bc}	18.0^{bc}	26.5^{a}	24.1^{a}	0.97
Total n3	3.9^{c}	6.2^{b}	9.2^{a}	9.7^{a}	1.9^{d}	1.8^{d}	2.3^{d}	2.3^{d}	0.19

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols;
PO = Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil; SO+T = Sunflower oil + Tocopherols
SAFA = Saturated fatty acids; MUFA = Monounsaturated fatty acids; PUFA = Polyunsaturated fatty acids.
SEM = Standard error of mean.
a-e Means within same row with no common superscripts differ significantly ($P<0.05$)

Table 4-2. Major Fatty Acid Compositions of Freeze-Dried Chicken Meat in Different Treatments After Four Months of Storage

Fatty acid (%)	Laying Hen Diets								
	FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T	SEM
C16:0	35.4^{ab}	36.3^{a}	34.5^{ab}	33.1^{b}	36.3^{a}	36.1^{a}	36.9^{a}	36.2^{a}	0.87
C18:0	8.2^{c}	9.4^{b}	9.5^{b}	9.1^{bc}	8.7^{bc}	8.3^{c}	11.1^{A}	10.6^{A}	0.35
C16:1	7.3^{a}	5.7^{b}	4.4^{cde}	5.1^{bc}	4.4^{cde}	5.0^{bcd}	3.7^{e}	4.1^{de}	0.34
C18:1	40.6^{ab}	39.8^{ab}	42.1^{a}	43.6^{a}	42.6^{a}	43.1^{a}	37.3^{b}	37.9^{b}	1.45
C18:2n6	1.8^{d}	2.4^{d}	3.9^{bc}	3.6^{c}	2.5^{d}	2.3^{d}	4.8^{a}	4.6^{ab}	0.30
C20:4n6	0.57^{d}	1.1^{bc}	0.98^{c}	1.0^{c}	1.4^{b}	1.2^{bc}	2.0^{a}	2.3^{a}	0.13
C18:3n3	0.2^{b}	0.2^{b}	0.4^{a}	0.4^{a}	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}	0.04
C20:5n3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
C22:5n3	0.1	0.2	0.1	0.1	0.0	0.0	0.0	0.0	0.03
C22:6n3	0.5^{b}	0.7^{a}	0.4^{c}	0.4^{c}	0.2^{e}	0.2^{e}	0.2^{de}	0.3^{cd}	0.04
SAFA	46.0^{abc}	47.8^{ab}	45.3^{bc}	43.5^{c}	46.3^{abc}	45.8^{bc}	49.1^{a}	48.0^{ab}	1.12
MUFA	48.2^{a}	45.8^{ab}	46.8^{a}	49.0^{a}	47.2^{a}	48.3^{a}	41.2^{c}	42.2^{bc}	1.45
Total n-6	2.5^{d}	3.6^{cd}	5.0^{b}	4.8^{bc}	4.1^{bc}	3.8^{bc}	7.3^{a}	7.3^{a}	0.45
Total n-3	0.8^{b}	1.1^{a}	0.8^{b}	0.8^{b}	0.2^{c}	0.2^{c}	0.2^{c}	0.4^{c}	0.08
PUFA	3.3^{d}	4.7^{bcd}	5.9^{b}	5.6^{bc}	4.4^{cd}	4.1^{d}	7.6^{a}	7.7^{a}	0.50

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols;
PO = Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil;
SO+T = Sunflower oil + Tocopherols
SAFA = Saturated fatty acids; MUFA = Monounsaturated fatty acids; PUFA = Polyunsaturated fatty acids.
SEM = Standard error of mean.
a-e Means within same row with no common superscripts differ significantly ($P<0.05$)

Table 4-3. Total Tocopherol Content Changes of Freeze-Dried Chicken Meat in Different Treatments during Storage

Storage	Laying Hen Diets								
(month)	FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T	SEM
	(μg/g meat powder)								
0	7.8^{c}	10.8^{b}	5.7^{d}	12.2^{ab}	6.6^{cd}	12.1^{b}	6.2^{d}	13.8^{a}	0.56
4	nd*	nd	nd	nd	nd	nd	nd	nd	

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols; PO = Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil; SO+T = Sunflower oil + Tocopherols.

a-b Means within same row with no common superscripts differ significantly ($P<0.05$)

* nondetectable

Table 4-4. Effects of Dietary Oils with and without Tocopherols and Storage on 2-Thiobarbituric Acid Values (TBA) of Freeze-Dried Chicken Meat

Storage	Laying Hen Diets							
(month)	FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T
TBA values (mg malonaldehyde per kg frozen meat)								
0	$13.3^{a, x}$	$5.1^{c, y}$	$6.7^{b, y}$	$3.8^{de, y}$	$3.7^{de, y}$	$2.3^{f, y}$	$4.3^{d, y}$	$3.4^{e, y}$
4	$13.0^{a, x}$	$12.1^{b, x}$	$8.6^{c, x}$	$8.9^{c, x}$	$7.1^{d, x}$	$7.2^{d, x}$	$7.2^{d, x}$	$7.6^{d, x}$

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil;
LO+T = Flax oil + Tocopherols;
PO = Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil;
SO+T = Sunflower oil + Tocopherols.
x-y Means within same column with no common superscripts differ significantly ($P<0.05$)
a-e Means within same row with no common superscripts differ significantly ($P<0.05$)
Pooled standard error of mean = 0.23.

Table 4-5. Changes of Lipid Classes in Freeze-Dried Chicken Meat Powder during Storage

Storage	Laying Hen Diets								
(month)	FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T	SEM
	Neutral lipids[1] (%)								
0	85.5a	66.2a	81.0a	77.3a	73.6a	77.0a	51.4a	62.5a	2.18
4	30.1b	20.6b	13.0b	16.5b	29.2b	26.5b	10.8b	11.9b	
	Polar lipids (%)								
0	14.6b	33.8b	19.0b	22.7b	26.4b	23.0b	48.6b	37.5b	2.20
4	69.7a	79.4a	87.0a	83.5a	70.8a	73.4a	89.2a	88.1a	

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols;
PO = Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil;
SO+T = Sunflower oil + Tocopherols
SAFA = Saturated fatty acids; MUFA = Monounsaturated fatty acids;
PUFA = Polyunsaturated fatty acids.
[1] Neutral lipids including triglycerides, diglycerides and cholesterol
SEM = Standard error of mean.
a-b Means within same column with no common superscripts differ significantly ($P<0.05$)

Table 4-6. Effects of Dietary Oils and Tocopherols and Storage on Cholesterol Oxidation in Freeze-Dried Chicken Meat

Diets	Total COPs	Major COPs* (ug/g) 7α-OH	7β-OH	α-Epoxide	β-epoxide	triol	7-keton
			Zero month storage				
FO	5.5^{a}	0.94^{a}	1.0^{a}	1.1^{a}	0.76^{abc}	0.0	1.8^{a}
FO+T	4.2^{ab}	0.89^{a}	0.79^{ab}	0.89^{abc}	0.46^{c}	0.0	1.2^{ab}
LO	4.6^{ab}	0.67^{ab}	0.57^{abc}	0.87^{abc}	0.82^{abc}	0.0	1.6^{ab}
LO+T	3.8^{ab}	0.63^{ab}	0.57^{abc}	0.98^{ab}	0.71^{abc}	0.0	0.94^{b}
PO	3.0^{b}	0.36	0.35^{bc}	0.34^{bcd}	1.3^{ab}	0.0	0.62^{b}
PO+T	3.1^{b}	1.0^{a}	1.0^{a}	0.03^{d}	0.3^{c}	0.0	0.77^{b}
SO	2.8^{b}	0.45^{b}	0.47^{bc}	0.19^{cd}	0.65^{bc}	0.0	1.1^{b}
SO+T	3.2^{b}	0.37^{b}	0.34^{c}	0.4^{abcd}	1.4^{a}	0.0	0.69^{b}
SEM	0.6	0.13	0.15	0.24	0.23		0.21
			Four month storage				
FO	256.8^{a}	38.8^{ab}	26.6^{abc}	12.7^{a}	71.4^{a}	3.7^{a}	103.6^{a}
FO+T	220.8^{ab}	34.8^{ab}	27.2^{abc}	11.6^{a}	62.4^{ab}	2.7^{a}	82.1^{ab}
LO	224.3^{ab}	35.3^{ab}	27.3^{abc}	11.8^{a}	64.6^{ab}	2.7^{a}	82.6^{ab}
LO+T	160.1^{c}	23.9^{b}	19.3^{c}	8.6^{ab}	48.0^{ab}	1.9^{a}	58.5^{c}
PO	189.2^{bc}	29.0^{ab}	23.3^{bc}	9.3^{a}	48.8^{ab}	1.8^{a}	77.0^{bc}
PO+T	154.0^{c}	38.6^{ab}	29.8^{ab}	4.9^{b}	21.9^{c}	1.9^{a}	56.9^{c}
SO	259.6^{a}	42.1^{ab}	33.6^{a}	12.7^{a}	66.8^{ab}	3.8^{a}	100.5^{a}
SO+T	196.0^{bc}	48.9^{a}	26.7^{abc}	8.9^{ab}	42.9^{bc}	5.0^{a}	63.8^{bc}
SEM	15.2	7.8	3.3	1.4	8.6	1.1	7.4

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols;
PO= Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil;
SO+T = Sunflower oil + Tocopherols.
7α-OH: 7α-Hydroxycholesterol; 7β-OH: 7β-Hydroxycholesterol; 7-Keto: 7-Ketocholesterol;
25-OH: 25 hydroxycholesterol;
* Cholesterol oxidation products
SEM = Standard error of mean
a-b Means within same column with no common superscripts differ significantly (P≤0.05)

Table 4-7. Effects of Dietary Oils and Tocopherols and Storage on The Loss of Cholesterol and Formation of its Oxides in Freeze-Dried Chicken Meat

Sterols Content	Storage (month)	FO	FO+T	LO	LO+T	PO	PO+T	SO	SO+T
Cholesterol (mg/g of yolk)	0	1.4±0.12*	1.4±0.11	1.4±0.14	1.4±0.12	1.5±0.12	1.4±0.13	1.5±0.12	1.5±0.15
	4	0.6±0.03	0.8±0.07	0.8±0.1	0.8±0.07	0.8±0.05	0.74±0.02	0.8±0.01	0.79±0.01
Total[1] COPs (ppm)	4	256.8±12	220.8±27	224.3±30	160.1±33	189.2±14	154.0±11	259.6±43	196.0±20
Total COPs[2]/ cholesterol (%)	4	18.4	15.8	16.0	11.4	12.6	11.0	17.1	13.1

FO = Fish oil; FO+T = Fish oil + Tocopherols; LO = Flax oil; LO+T = Flax oil + Tocopherols;
PO = Palm oil; PO+T = Palm oil + Tocopherols; SO = Sunflower oil; SO+T = Sunflower oil + Tocopherols.

[1] Total cholesterol oxides

[2] Cholesterol oxidation products

* Mean ± SD (n=3)

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Chapter 5. General Conclusions

Cholesterol is ubiquitously present in foods, of animal origin such as meat and egg products. Cholesterol is quite unstable, and easily oxidized to form cholesterol oxidation products (COPs) when exposed to air, heat, or light. The major COPs in foodstuffs include the following: 25-hydroxycholesterol (25-OH), cholestan-3β-5α-6β-triol (triol), 5,6α-epoxy-cholesterol (α-epoxide), 5,6β-epoxycholesterol (β-epoxide), 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH), and 7-ketocholesterol (7-keto) (Finocchiaro & Richardson, 1983). Of these COPs 25-OH and triol have been shown to be the most toxic to cultured cells and experimental animals (Kandutsch et al., 1978; Peng et al, 1979; Imai et al., 1980; Ansari et al., 1982).

Newly developed foodstuffs are often composed of ingredients that have been processed, dried, or stored before use, and one may wonder whether this increases cholesterol oxides in the diet. Cholesterol oxides display angiotoxicity at very low doses: Jacobson et al. (1985) have demonstrated that pigeons fed a diet containing 0.5% cholesterol and cholestanetriol at 0.3% of cholesterol by weight had more coronary stenosis and calcium accumulation in the aortic tissues than pigeons fed a diet containing 0.5% cholesterol alone. Therefore, the scientific study of autoxidation of cholesterol in food has become a critical research needs.

In this thesis, results of the studies conducted showed that cholesterol is easily oxidized during storage and elevated temperature in vitro and in vivo. Fatty acid

composition and tocopherol content in food oils influenced the stability of cholesterol. Fish oil was easily oxidized. Level of oxides in oils was also increased by lengthy storage and cholesterol oxidation is accelerated by the presence of highly long chain polyunsaturated fatty acids, whereas tocopherols effectively delaying cholesterol oxidation. Vegetable oils intrinsically contain tocopherols. Therefore, the benefits of adding extra tocopherols to these oils seem marginal. Products from sources that are low or deficient in natural antioxidants like fish oil can be improved by tocopherol supplementation.

Yolk and chicken meat fatty acids can be easily altered by dietary manipulation (Cruickshank, 1934; Ajuyah et al 1991a,b). Egg yolk powder and meat from laying hens fed fish oil were easily oxidized and were significantly higher levels of oxides than palm oil diet treatments. This research indicates that cholesterol oxidation is accelerated by the presence of highly long chain polyunsaturated fatty acids. High temperature has been recognized as a strong factor in lipid autoxidation. Deep fat frying is a common method of food preparation in restaurants and in processing plants. During frying, the heating medium may experience abusive conditions due to repeated exposure to oxygen at elevated temperatures (Park and Addis, 1986). The thermal oxidation and polymerization of fatty acids, including toxicological implications and loss of nutritional value have been reported (Kaunitz, 1967; Chang et al., 1978; Thompson and Aust, 1983). The results of this study suggest that high temperature apparently increased cholesterol oxide formation, and tocopherol supplementation can decreased cholesterol oxide content in food oils and poultry products. 7-Ketocholesterol and β-epoxide were predominant compounds. Feeding tocopherol supplemented diets to laying hen resulted in a dependent increase of the

tocopherol addition in the dried egg yolk powder and meats. The prolonged storage of the egg powders led to a gradual ($P<0.05$) decline in total tocopherol contents with time over the four month period and after cooking. The extent of the decline was positively correlated with the initial concentration of tocopherols in the powder, with the greatest losses occurring in powders with the highest initial content. Cholesterol oxide contents in yolk powder with tocopherol were 30% less than in egg yolk powder without tocopherol from fish oil and flax oil diets. However, no significant differences were observed in chicken meat from sunflower, flax, palm oils with and without tocopherol treatments, and no detectable tocopherols were presented in chicken meat after 4 month storage. Tocopherols supplementation to hen diet resulted in improved oxidative cholesterol stability. This indicates the potential for a supplementation program in food-producing animals in order to produce higher-quality meat and meat products and protect the consumer from the potentially deleterious biological effects of lipid oxidation products.

Iatroscan TLC-FID lipid analysis showed that percent of polar lipids increased with a decrease in neutral lipids with long storage at room temperature. Changes in neutral and/or polar lipid content could be used as indicators of lipid oxidation in foods.

In conclusion, cholesterol oxidation can be accelerated by the presence of highly long chain polyunsaturated fatty acids, high temperature and long term storage. Tocopherol supplementation can successfully decrease cholesterol oxide formation in foods. Methods for preventing cholesterol oxide formation in commercially prepared and stored foods by simply increasing the intrinsic tocopherol concentration of eggs and

meats, which are a major source of cholesterol, would benefit the food industry and human health.

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